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(54) NOVEL RIG-I LIGANDS AND METHODS FOR PRODUCING THEM

NEUARTIGE RIG-I-LIGANDEN UND HERSTELLUNGSVERFAHREN DAFÜR

NOUVEAUX LIGANDS DE RIG-I ET PROCÉDÉS POUR LES PRODUIRE

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(73) Proprietor: **Rheinische Friedrich-Wilhelms-Universität Bonn**
53113 Bonn (DE)

(72) Inventors:
- **GOLDECK, Marion**
  1030 Wien (AT)
- **VAN DEN BOORN, Jasper**
  241 WJ Bodegraven (NL)
- **LUDWIG, János**
  37085 Göttingen (DE)
- **SCHUBERTH-WAGNER, Christine**
  82256 Fürstenfeldbruck (DE)

(74) Representative: **Wichmann, Hendrik**
Wuesthoff & Wuesthoff
Patentanwälte PartG mbB
Schweigerstraße 2
81541 München (DE)

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- **MARTIN SCHLEE ET AL: "Recognition of 5' Triphosphate by RIG-I Helicase Requires Short Blunt Double-Stranded RNA as Contained in Panhandle of Negative-Strand Virus", IMMUNITY, vol. 31, no. 1, 1 July 2009 (2009-07-01), pages 25-34, XP055032801, ISSN: 1074-7613, DOI: 10.1016/j.immuni.2009.05.008**
- **YANLI WANG ET AL: "Structural and functional insights into 5'-ppp RNA pattern recognition by the innate immune receptor RIG-I", NATURE STRUCTURAL & MOLECULAR BIOLOGY, vol. 17, no. 7, 1 July 2010 (2010-07-01), pages 781-787, XP055052657, ISSN: 1545-9993, DOI: 10.1038/nsmb.1863**

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Description

[0001] The present invention relates to new modified oligonucleotides which may act as RIG-I ligands as well as to pharmaceutical compositions comprising same, as further defined in the claims.

Background of the invention

[0002] Schlee et al., Immunity, 2009, 31, 25-34 describe blunt-ended double stranded RNAs carrying a 5'-O-triphosphate moiety on one of the strands that act as potent stimulators of the immune system by binding the RIG-I helicase. Thus, there is a need to provide a simple and efficient method for preparing triphosphate-modified oligonucleotides in high purity, suitable for pharmaceutical applications.

[0003] The coupling of triphosphate groups or analogues thereof to the 5'-OH group of nucleosidic compounds is well known in the art. Ludwig J. et al., J. Org. Chem., 1989, 54, 631-635 disclose a solution triphosphorylation method for preparing 5'-O-triphosphates of nucleosides and analogues using 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one as the phosphitylating agent. Gaur R.K. et al., 1992, Tetrahedron Letters, 33, 3301-3304 describe the use of said method on solid-phase for the synthesis of 2'-O-methylribonucleoside 5'-O-triphosphates and their P_{α}-thio analogues. US-Patent 6,900,308 B2 discloses the solid-phase synthesis of modified nucleoside 5'-O-triphosphates as potential antiviral compounds and US-Patents 7,285,658, 7,598,230 and 7,807,653 disclose triphosphate analogues of nucleosides with modifications in the sugar, nucleobase and in the triphosphate entity.

[0004] WO96/40159 describes a method for producing capped RNA or RNA analogue molecules, wherein an RNA or RNA analogue oligonucleotide is reacted with a phosphitylating agent such as 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one or a ring-substituted derivative thereof. The resulting intermediate is reacted with a phosphate or pyrophosphate or salt thereof, oxidized or hydrolyzed. The di- or triphosphorylated RNA or RNA analogue is capped by reacting with an activated m^7G tri-, di- or monophosphate or analogue.

[0005] WO 2009/060281 describes immune stimulatory oligoribonucleotide analogues containing modified oligophosphate moieties and methods for the preparation of such compounds. This method includes the synthesis of the oligonucleotide on a solid support, reacting a nucleotide at a 5'-end of the oligonucleotide with a phosphitylating agent such as 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in a suitable solvent and in the presence of a base, reacting the phosphitylated oligonucleotide with a pyrophosphate or pyrophosphate analogue, oxidizing the oligonucleotide with an oxidizing agent and deprotecting the oligonucleotide to give a triphosphate- or triphosphate analogue-modified oligonucleotide.

[0006] Polyacrylamide gel-electrophoresis as employed in WO 96/40159 is applicable only for small scale separations. The resolution power of ion exchange chromatography for 5'-mono-, di-, triphosphorylated products of longer oligoribonucleotides is limited. The required denaturing conditions make separation a tedious task (Sproat, 1999; Zlatev, 2010; WO 2009/060281), moreover, products are usually contaminated with n-1, n-2 sequences and their mono- and diphosphates resulting in insufficient purity. Given the sensitivity for precise terminal structures of the RIG-I ligands, these purification methods are suboptimal for pharmacological applications. The European Patent Office *inter alia* identified the prior art document Wang Y. et al. (2010) published in Nature Structural & Molecular Biology 17(7), p.781-787, which discloses structural and functional insights into 5'ppp RNA pattern recognition by the innate immune receptor RIG-I.

[0007] Thus, there is a high need for new triphosphorylated oligonucleotides and analogues thereof, in particular having RIG-I selectivity as well as methods for preparing such compounds.

[0008] The present invention relates to novel oligonucleotides which can be produced in large scales for potential clinical use, as defined in the claims.

[0009] Specifically, the present invention relates to an oligonucleotide comprising a sense strand and an antisense strand, wherein the oligonucleotide comprises

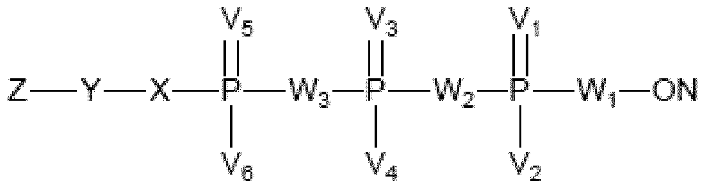
a sense strand comprising nucleotide sequence
5' GACGCUGACCCUGAA$_m$GUUCAUCUU 3'; and
an antisense strand comprising nucleotide sequence
3' CUG$_m$CGACUGGGACUUCAAGUAGAA 5';

wherein the nucleotide indexed m is 2'-O-methylated, as defined in the claims.

[0010] Furthermore, the present invention relates to a pharmaceutical composition comprising an oligonucleotide of the present invention, as defined in the claims.

[0011] A convenient preparation method for such oligonucleotides is also disclosed. Furthermore, modifications of oligonucleotides are described which establish, maintain and/or improve the RIG-I selectivity of the oligonucleotides or enhance their chemical stability.

[0012] Thus, a first aspect of the present disclosure relates to a modified oligonucleotide of Formula (I)



$$Z-Y-X-\underset{V_6}{\overset{V_5}{\overset{\|}{\underset{|}{P}}}}-W_3-\underset{V_4}{\overset{V_3}{\overset{\|}{\underset{|}{P}}}}-W_2-\underset{V_2}{\overset{V_1}{\overset{\|}{\underset{|}{P}}}}-W_1-ON \qquad \mathrm{I}$$

wherein V_1, V_3 and V_5 are independently in each case selected from O, S and Se;

V_2, V_4 and V_6 are independently in each case selected from OH, OR^1, SH, SR^1, F, NH_2, NHR^1, $N(R^1)_2$ and $BH_3^-M^+$,
W_1 is O or S,
W_2 is O, S, NH or NR^2,
W_3 is O, S, NH, NR^2, CH_2, CHHal or $C(Hal)_2$,
R^1, R^2 and R^3 are selected from C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{2-6} acyl or a cyclic group, each optionally substituted, or wherein two R^1 may form a ring together with an N-atom bound thereto,
M^+ is a cation,
X is NH, NR^3, O or S,
Z represents a capture tag or H,
Y represents a bond or a linker connecting the capture tag to X, and
ON represents an oligonucleotide comprising at least 4 nucleotide or nucleotide analogue building blocks.

[0013] The term "oligonucleotide", as used in the context, encompasses compounds comprising a plurality, e.g. at least 4 nucleotide or nucleotide analogue building blocks. Preferably, the oligonucleotide comprises 6-100, e.g. 20-40 building blocks. The nucleotide or nucleotide analogue building blocks may comprise nucleoside or nucleoside analogue subunits connected by inter-subunit linkages. The nucleoside subunits include deoxyribonucleoside subunits, ribonucleoside subunits and/or analogues thereof, particularly sugar- and/or nucleobase-modified nucleoside analogues. Further, the oligonucleotides may comprise non-nucleotidic building blocks and/or further terminal and/or side-chain modifications.

[0014] In preferred sugar-modified subunits the 2'-OH of a ribonucleoside subunit is replaced by a group selected from OR, R, halo, SH, SR, NH_2, NHR, NR_2 or CN, wherein R is C_{1-6} alkyl, C_{2-6} alkenyl or C_{2-6} alkynyl and halo is F, Cl, Br or I. In further preferred sugar-modified subunits, the ribose may be substituted, e.g. by another sugar, for example a pentose such as arabinose. This sugar modification may be combined with 2'-OH modifications as described above, such as in 2'-fluoroarabinonucleoside subunits. Still further preferred sugar-modified subunits include locked nucleosides (LNA) or 2',3'-seco- nucleosides (UNA). In preferred nucleobase-modified nucleosidic building blocks, a non-standard, e.g. non-naturally occurring nucleobase, is used instead of a standard nucleobase. Examples of non-standard nucleobases are uracils or cytosines modified at the 5-position, e.g. 5-(2-amino)propyl uracil or 5-bromouracil; hypoxanthine; 2,6-diaminopurine; adenines or guanines modified at the 8-position, e.g. 8-bromoguanine; deazanucleosides, e.g. 7-deazaguanine or 7-deazaadenine; or O- and N-alkylated nucleobases, e.g. N^6-methyladenine, or N^6,N^6-dimethyladenine. Further suitable nucleobase analogues may be selected from universal nucleobase analogues such as 5-nitroindole.

[0015] The inter-subunit linkage between subunits may be a phosphodiester linkage or a modified linkage, e.g. a phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, boranophosphate, or another modified linkage known to a skilled person in the art.

[0016] The oligonucleotide may be selected from deoxyribonucleotides, ribonucleotides and oligonucleotide analogues. The deoxyribonucleotides, ribonucleotides and/or oligonucleotide analogues may be chemically modified at the nucleoside and/or ribose subunit of the analogue, deoxyribonucleotide, ribonucleotide and/or oligonucleotide. Analogues of desoxyribonucleotides or ribonucleotides may comprise at least one desoxyribonucleoside or ribonucleoside subunit and at least one modified nucleosidic subunit and/or at least one modified inter-subunit linkage, e.g. as described above. Oligonucleotide analogues may also consist in their entirety of modified nucleosidic subunits.

[0017] The oligonucleotide is a double-stranded molecule, as defined in the claims. Double-stranded oligonucleotides may comprise completely or partially complementary strands. Double-stranded molecules may be blunt-ended or comprise at least one overhang, e.g. a 5'- or 3'-overhang. Overhangs, if present, are preferably located at the distal end of the molecule (with regard to the triphosphate/triphosphate analogue group). Double-stranded oligonucleotides may also comprise a hairpin-structure, wherein the duplex is closed by a loop at the distal end thereof (with regard to the triphosphate/triphosphate analogue group). The loop may comprise nucleotide and/or non-nucleotide building blocks, for example diol-based building blocks such as ethylene glycol moieties, e.g. tri(ethylene)glycol or hexa(ethylene)glycol; propane-1,3-diol; dodecane-1,12-diol; or 3,12-dioxa-7,8-dithiatetradecane-1,14-diol.

[0018] In a preferred embodiment, double-stranded molecules are blunt-ended, particularly at the proximal end thereof (with regard to the triphosphate/triphosphate analogue group).

[0019] The oligonucleotide is double-stranded, wherein each strand of the double strand has a length of at least 19 nucleotides, as defined in the claims. A blunt-ended double-stranded oligonucleotide of such a length is especially preferred. According to a further preferred embodiment each strand of the oligonucleotide has a length of at least 19 to 50 nucleotides, 19 to 30 nucleotides, 20 to 30 nucleotides, 22 to 28 nucleotides, especially preferred 22 to 26 nucleotides.

[0020] The oligonucleotide may comprise further terminal and/or side-chain modifications, e.g. cell specific targeting entities covalently attached thereto. Those entities may promote cellular or cell-specific uptake and include, for example lipids, vitamins, hormones, peptides, oligosaccharides and analogues thereof. Targeting entities may e.g. be attached to modified nucleobases or non-nucleotidic building blocks by methods known to the skilled person.

[0021] According to a preferred embodiment modifications establish and/or enhance the selectivity of the oligonucleotide towards a given target. In a particularly preferred embodiment the RIG-I selectivity of the oligonucleotide is established or enhanced. Methods to determine the RIG-I selectivity of a given oligonucleotide are described herein in detail (cf. Examples) and/or are known to the person skilled in the art.

[0022] According to another preferred embodiment the chemical modifications maintain or enhance the chemical stability of the oligonucleotide. A person skilled in the art knows methods for determining the chemical stability of a given oligonucleotide. Such methods are also described, e.g., in the Examples.

[0023] According to a preferred embodiment the chemical modifications of the oligonucleotide are selected from the group comprising halogenation, in particular F-halogenation, 2'-O-alkylation, in particular 2'-O-methylation, and/or phosphorothioate modifications of internucleotide linkages, as defined in the claims. Particularly F-halogenation and phosphorothioate modifications increase the stability of the oligonucleotide, while 2'-O-methylation establishes or increases RIG-I selectivity of the oligonucleotide. 2'-O- methylations are also able to modify the immunogenicity of RNA. As further defined in the claims, an oligonucleotide comprises one 2'-O-methylation per strand.

[0024] The 2'-F substitution is particularly preferred. At the 2' position of the ribose the hydroxyl group is substituted for fluoro. 2'-F substitutions in RNAs particularly result in an enhanced stability against nuclease digestion. In a further embodiment, a 2'-fluoro-substitution may particularly augment a RIG-I-dependent immune stimulation.

[0025] Herein, phosphorothioate compounds in general relate to phosphorothioate modifications of internucleotid linkages.

[0026] Phosphorothioate-modified compounds having a modification at a terminal end of the oligonucleotide are especially preferred. During phosphorothioate modification the non-binding oxygen atom of the bridging phosphate is substituted for a sulfur atom in the backbone of a nucleic acid. This substitution reduces the cleavability by nucleases at this position significantly and results in a higher stability of the nucleic acid strand.

[0027] In an especially preferred embodiment an oligonucleotide according to the present invention shows F-halogenation, methylation, in particular 2'-O-methylation, as well as phosphorothioate modifications, in particular at a terminal end of the oligonucleotide, as defined in the claims.

[0028] The identification patterns of a given oligonucleotide depend on the sequence and the length of an oligonucleotide and can be determined for each given oligonucleotide. A person skilled in the art is well aware how to carry out this determination.

[0029] As explained above already, such methods for determining RIG-I selectivity and/or stability of a given oligonucleotide are described in detail in the present disclosure.

[0030] The oligonucleotide of disclosed formula (I) or (IV) comprises a triphosphate/triphosphate analogue group. In this group, V_1, V_3 and V_5 are independently selected from O, S and Se. Preferably, V_1, V_3 and V_5 are O. V_2, V_4 and V_6 are in each case independently selected from OH, OR^1, SH, SR^1, F, NH_2, NHR^1, $N(R^1)_2$ and $BH_3^-M^+$. Preferably, V_2, V_4 and V_6 are OH. R^1 may be C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{2-6} acyl or a cyclic group, e.g. a C_{3-8} cyclo(hetero)alkyl group, a C_{3-8} cyclo(hetero)alkenyl group, phenyl or C_{5-6} heteroaryl group, wherein heteroatoms are selected from N, O and S. Further, two R^1 may form a ring, e.g. a 5- or 6-membered ring together with an N-atom bound thereto. R^1 may also comprise substituents such as halo, e.g. F, Cl, Br or I, O(halo)C_{1-2} alkyl and - in the case of cyclic groups - (halo)C_{1-2} alkyl. M^+ may be an inorganic or organic cation, e.g. an alkali metal cation or an ammonium or amine cation.

[0031] W_1 may be O or S. Preferably, W_1 is O. W_2 may be O, S, NH or NR^2. Preferably, W_2 is O. W_3 may be O, S, NH, NR^2, CH_2, CHHal or $C(Hal)_2$. Preferably, W_3 is O, CH_2 or CF_2. R^2 may be selected from groups as described for R^1 above. Hal may be F, Cl, Br or I.

[0032] According to an especially preferred instance of the disclosure V_1, V_2, V_3, V_4, V_5, V_6, W_1, W_2 and W_3 are O.

[0033] The triphosphate/triphosphate analogue group is preferably attached to a terminus of the oligonucleotide. Preferably, the group is attached to the 5'-terminus of the oligonucleotide, particularly to the 5'-OH-group of the 5'-terminal sugar thereof.

[0034] As defined herein, Z represents a capture tag or H. The capture tag Z can be functionally defined by a series of plausible examples as presented below. A general rule may be: Z has to allow a convenient purification and it should be removable under conditions which are compatible with pppRNA stability requirements. A person skilled in the art is able to determine without undue burden whether a given tag fulfils the functional definition or not. Thus, a person skilled in the art is aware of such capture tags, in particular with regard to the detailed examples given in the present application.

[0035] According to a preferred instance of the disclosure the capture tag Z is selected from a long-chain aliphatic residue, a partner of a non-covalent high-affinity binding pair, a reactive chemical entirety, Q or NHC$_2$-C$_{24}$alkyl, Q being preferably selected from H, amino acids, amino acid analogues, C$_1$-C$_{24}$alkyl, preferably C$_{12}$-C$_{24}$alkyl, peptides and lipids. However, according to an especially preferred instance of the disclosure Z is decyl, i.e. C$_{10}$ alkyl.

[0036] The capture tag Z according to the present disclosure is a moiety capable of non-covalently or covalently interacting with a capture reagent under conditions which allow separation for compounds comprising the capture tag, e.g. the oligonucleotide (I) from other species, which do not contain the capture tag. Preferably, the capture reagent is an immobilized reagent or a reagent capable of being immobilized.

[0037] Suitable capture tags are for instance long-chain, e.g. C8-24, preferably C13-24, more preferably C13-C14 aliphatic alkyl residues such as octadecyl or other lipidic/lipophilic residues such as e.g. cholesteryl, tocopheryl or trityl and derivatives thereof. However, according to an especially preferred instance of the disclosure Z is a decyl residue. In this case, the tagged triphosphate entity can be captured and purified on a solid phase by standard reversed phase chromatography, e.g. RP-HPLC, or by hydrophobic interaction chromatography (HIC). The capture tag may also be a perfluoroalkyl entity, e.g. a 4-(1H,1H,2H,2H-perfluorodecyl)benzyl or a 3-(perfluorooctyl)propyl residue for specific capture of the modified oligo-triphosphate on a Fluorous Affinity support such as is commercially available from Fluorous Technologies, Inc.

[0038] In another instance of the disclosure, the capture tag may be a first partner of a non-covalent high-affinity binding pair, such as biotin, or a biotin analogue such as desthiobiotin, a hapten or an antigen, which has a high affinity (e.g. binding constant of 10-6 l/mol or less) with the capture reagent, which is a second complementary partner of the high-affinity binding pair, e.g. a streptavidin, an avidin or an antibody.

[0039] In yet another instance of the disclosure, the capture tag may be a first partner of a covalent binding pair, which may form a covalent bond with the capture reagent, which is a second complementary partner of the covalent binding pair, wherein the covalent bond may be a reversible or an irreversible bond. In this embodiment, the capture tag component Z may be a reactive chemical entity such as an azide or alkynyl group enabling covalent reaction with a capture reagent that contains a complementary reactive group, e.g. an alkynyl or azido moiety, respectively, in the case of the Husigen 3+2 cycloaddition reaction (the so-called "click-reaction" that is Cu(I) catalyzed or a variant thereof that proceeds without Cu(I) ions via release of severe ring strain in e.g. cyclooctyne derivatives). A specific example for Z-Y-X in such a case would be propargylamino.

[0040] In another instance of the disclosure, the capture tag component may be a chemical entity which contains an additional nucleophilic group, for instance a second amino group in an NH2-Y-XH type reagent. A wide range of suitable electrophilic Z reagent such as cholesterol, chloroformiate or biotin N-hydroxy succinimide active esters may then be used to introduce the tagging group while the oligonucleotide is attached to the solid phase, thus significantly extending the scope of the tagging reaction.

[0041] In a preferred instance of the disclosure the capture tag is a long-chain alkyl residue, a perfluoroalkyl entity, an azide or an alkynyl group.

[0042] In a further instance of the disclosure, the oligonucleotide may carry a second capture tag at a different position, e.g. at the 3'-terminus. The first and the second capture tags are preferably selected as to allow purification by two orthogonal methods to enable recovery of extremely high purity material. For example the first capture tag may be a lipophilic group, which interacts with a suitable chromatographic support and the second capture tag may be biotin, which interacts with streptavidin.

[0043] The second capture tag may be conveniently introduced by performing the synthesis using a modified CPG (controlled glass support) for oligoribonucleotide synthesis.

[0044] Y represents a chemical bond or a linker, e.g. an alkylene, preferably a C1-6-alkylene linker, more preferably a C2-5-alkylene linker, or aralkylene linker, optionally comprising heteroatoms or heteroatom-containing groups, such as O, S, NH, C=O or C=S, and/or optionally comprising C=C or C=C bonds. According to an especially preferred embodiment Y is a bond.

[0045] In another preferred instance of the disclosure the linker is a polyalkylene oxide, preferably a poly-C2-C6-alkylene oxide, more preferably a poly-C2-C3-alkylene oxide. The number average molecular weight of the linker may be in the range from 30-800 g/mol, preferably from 40-450 g/mol, more preferably from 40-250 g/mol. The linker may be [-CH2CHR4-O-]n with n = 1-10, preferably n = 1-7, more preferably n = 2-5, and even more preferably n = 3. R4 may be H or C1-6-alkyl. Further preferred embodiments of Y are shown in Figure 4. In a preferred embodiment R4 is H.

[0046] According to an especially preferred instance of the disclosure

X is NH or O,
Y is -K-((CHR$_1$)$_m$-CH$_2$-O)$_n$-R-, or
(O-(CHR$_3$)$_{m3}$-CH$_2$)$_{n1}$-(O-(CHR$_2$)$_{m2}$-CH$_2$)$_{n2}$-(O-(CHR$_1$)$_{m1}$-CH$_2$)$_{n3}$-, and K is O or NH,
m, m$_1$, m$_2$ and m$_3$ is independently 1 to 12, preferably 1 to 8, more preferably 1 to 5, and even more preferably 1 to 3,
n, n$_1$, n$_2$ and n$_3$ is independently 0 to 20, preferably 0 to 10, more preferably 0 to 5, and even more preferably 0 to 3, and
R$_1$, R$_2$ and R$_3$ is independently is H, C$_{1-6}$ alkyl, C$_{2-6}$ alkenyl, C$_{2-6}$ alkynyl,

C_2- C_6-acyl or a cyclic group, each optionally substituted and
R is C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_2- C_6-acyl or a cyclic group, each optionally substituted. Preferably, R is CH_2CH_2.

[0047] According to an especially preferred instance of the disclosure with Y being as defined above, R^1 and R^2 are H, n_1 is O and n_2 and n_3 are 1. Further preferred embodiments can be taken from Figure 4.

[0048] According to another preferred instance of the disclosure as Y is being defined above, R_1, R_2 and R_3 are H and n_1, n_2 and n_3 are 1.

[0049] According to a preferred instance of the disclosure X is NH, K is NH and Y is $(CH_2CH_2O)_n$ with n as being defined above, wherein K is further substituted with cholesterol-C(O)-, trityl or derivatives thereof.

[0050] According to an especially preferred instance of the disclosure of the oligonucleotide according to Formula (I) X is NH or O, Y is a bond, and Z is C_1-C_{12}alkyl or H, preferably C_{10}, Q or NHC_2-C_{24}alkyl, wherein Q is selected from H, amino acids, amino acid analogues, C_1-C_{24}alkyl, preferably C_{12}-C_{24}alkyl, peptides and lipids, and V_1, V_2, V_3, V_4, V_5, V_6, W_1, W_2 and W_3 are O.

[0051] According to a further preferred instance of the disclosure of the oligonucleotide of Formula (I) X is NH or O, Y is a bond, and Z is decyl or H, and V_1, V_2, V_3, V_4, V_5, V_6, W_1, W_2 and W_3 are preferably O.

[0052] A further aspect of the present invention relates to a pharmaceutical composition comprising a modified oligonucleotide as defined in the claims.

[0053] The pharmaceutical composition according to the invention may further comprise pharmaceutically acceptable carriers, diluents, and/or adjuvants, as defined in the claims. The term "carrier" when used herein includes carriers, excipients and/or stabilisers that are non-toxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carriers an aqueous pH buffered solutions or liposomes. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate and other organic acids (however, with regard to the formulation of the present invention, a phosphate buffer is preferred); anti-oxidants including ascorbic acid, low molecular weight (less than about 10 residues) polypeptides; proteins such as serum albumin, gelatine or immunoglobulins; hydrophilic polymers such as polyvinyl pyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose or dextrins, gelating agents such as EDTA, sugar, alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or non-ionic surfactants such as TWEEN, polyethylene or polyethylene glycol. According to an especially preferred embodiment the compound of the invention is dissolved in sterile deionized water.

[0054] Such a composition and/or formulation according to the invention can be administered to a subject in need thereof, particularly a human patient, in a sufficient dose for the treatment of the specific conditions by suitable means, as defined in the claims. For example, the composition and/or formulation according to the invention may be formulated as a pharmaceutical composition together with pharmaceutically acceptable carriers, diluents and/or adjuvants, as defined in the claims. Therapeutic efficiency and toxicity may be determined according to standard protocols. The pharmaceutical composition may be administered systemically, e.g. intraperitoneally, intramuscularly, or intravenously or locally such as intranasally, subcutaneously, intradermally or intrathecally, as defined in the claims. The dose of the composition and/or formulation administered will, of course, be dependent on the subject to be treated and on the condition of the subject such as the subject's weight, the subject's age and the type and severity of the disease or injury to be treated, the manner of administration and the judgement of the prescribing physician.

[0055] In a preferred embodiment the pharmaceutical composition is administered intradermally. It is especially preferred that the composition is administered intradermally via tattooing, microneedling and/or microneedle patches, as defined in the claims.

[0056] The compound according to the present invention is preferably dissolved and diluted to the desired concentration in sterile, deionized water (purified water) and is then applied on the shaved, ethanol-disinfected skin using a pipetting device, and subsequently tattooed into the skin, as defined in the claims.

[0057] For tattooing, for example, the water-based pharmaceutical composition according to the invention is intradermally injected into the skin, using a (medical) tattoo device fitted with a multi-needle (single use) needle-tip (such as a 9-needle, single-use tip), as defined in the claims.

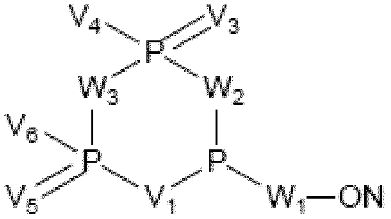
[0058] The typical tattooing procedure is as follows: After the water-based pharmaceutical composition is pipetted onto the shaved and ethanol cleaned skin, it is introduced into the tattoo machine's multi-needle tip by placing the running needle tip (running at a speed of, for example, 100-120 Hz, in particular at 100 Hz) gently on top of the droplet of water-based pharmaceutical composition. Once the droplet of water-based pharmaceutical composition is completely adsorbed in the running needle tip, and hence resides in between the running needles, the running tip is gently moved back and forth over the skin, by holding the now filled needle tip in an exact 90 degree angle to the skin. Using this method, the water-based pharmaceutical composition is completely tattooed into the skin. For 50-100 μl of water-based pharmaceutical composition this typically takes 10-15 seconds, over a skin area of 2-4 square centimeters. The benefit of this treatment over standard single intradermal bolus injection, is that the water-based pharmaceutical composition is evenly injected over a larger area of skin, and is more evenly and more precisely divided over the target tissue: By using a 9-needle tip at 100Hz for 10 seconds, this method ensures 9000 evenly dispersed intradermal injections in the treated skin.

[0059] Of course, a person skilled in the art may deviate from and adjust the procedure, depending on the patient or part of the body to be treated. The microneedling procedure may be carried out in close analogy to the tattooing procedure. However, with microneedling the tattoo needle-tip is replaced by a microneedling tip, which ensures more superficial intradermal administration. The water-based pharmaceutical composition is in principle pipetted onto the shaved and ethanol cleaned skin and then administered intradermally using the microneedling tip, in analogy to the tattoo procedure. Microneedling does not have necessity to prior adsorption of the pharmaceutical composition in between the microneedling needles.

[0060] Additionally, it is envisioned that microneedle patches coated with, or otherwise harbouring, the pharmaceutical composition can be used for transdermal/intradermal delivery. This has the specific advantage that the intradermal delivery of the pharmaceutical composition can be carried out safely by the recipient him-/herself in without the need for a hospital visit and/or medical specialist tattooing/microneedling intervention. This can significantly add flexibility to treatment schemes, allow highly personalized treatment regimens, lower treatment-associated pain, and lower treatment cost. These patches can be constituted of, but not be limited to, dissolving- or non-dissolving microneedle patches for the time-controlled-, sustained- or bolus transdermal delivery of the pharmaceutical composition.

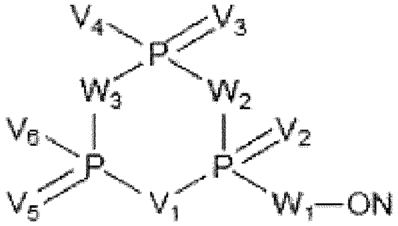
[0061] Another aspect of the present disclosure relates to a method of preparing an oligonucleotide, comprising the steps

(a) reacting a compound of formula (IIa)



IIa

wherein V1, V3, V5, V4, V6, W1, W2, W3, and ON are as defined above,
wherein ON is protected by at least one protection group, with an oxidizing agent to obtain a compound of formula (IIb)



IIb

wherein V1, V3, V5, V2, V4, V6, W1, W2, W3 and ON are as defined above,
wherein ON is protected by at least one protection group,
(b) reacting a compound of formula (IIb) with a capture tag agent of formula (III),

Z-Y-XH (III),

wherein X, Z, and Y are as defined above, wherein X is preferably O, to obtain a reaction product comprising the oligonucleotide of formula (I), and
(c) deprotection of the at least one ON protection group, and
(d) contacting the reaction product of step (c) with a capture reagent capable of interacting with the capture tag, wherein the contacting takes place under conditions which allow separation of the oligonucleotide (I) from other species contained in said reaction product.

[0062] An instance of the disclosure, wherein X is O, is especially preferred.

[0063] During the disclosed method the ON oligonucleotide comprises at least one protection group. The use of protective groups aims in particular at protecting the 2'-OH groups ribose subunit of the applied oligonucleotide. A person skilled in the art knows which protection groups are suitable for synthesis, especially a person working in the field of nucleotide synthesis. Protective groups at the 2'-OH position of the ribose subunit of the oligonucleotide are preferred. In a preferred instance of the disclosure , at the 2'-position of the ribose unit fluoride-labile protective groups are used.

[0064] Especially preferred are 2'-O-TBDMS or 2'-O-TOM protective groups. In a particularly preferred instance of the disclosure the TBDMS protective group is applied.

[0065] Particularly during the synthesis of compounds with X = O, which have an enhanced Z-Y-X-PPP binding stability, a broad spectrum of deprotection conditions may lead to the 2'-OH protective groups being cleaved off.

[0066] All known deprotection reagents are suitable to cleave off the TBDMS protective group. In particular, the following reagents may be applied:

(a) triethylamine-trihydrofluoride optionally in combination with a polar solvent,
(b) trialklylamine, triethylamine-trihydrofluoride and a polar solvent,
(c) pyridine-HF and other adducts of hydrofluoride of organic nitrogen bases,
(d) ammonium fluoride,
(e) tetra-n-butyl-ammonium fluoride,
(f) tetramethyl-ammonium fluoride, and other tetraalkyl-ammonium fluorides and combinations thereof.

[0067] Step (c) is preferably carried out under conditions which do not cause degradation of the triphosphate moiety, e.g. as described in detail below.

[0068] Step (a) of the disclosed method comprises the reaction of cyclic P(V)-P(V)-P(III) species of formula (IIa) with an oxidizing agent. The compound of formula (IIa)
may be obtained according to standard methods as described by Ludwig et al, 1989, supra and Gaur et al., 1992, supra, namely by reacting the 5'-terminal OH-group of an oligonucleotide with a trifunctional phosphitylating agent, e.g. 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one under suitable conditions, e.g. in the presence of base (pyridine or diisopropylmethylamine) in a suitable solvent such as dioxane or dichloromethane, and subsequent reaction with pyrophosphate (W_3=O) or a modified pyrophosphate (W_3 is different from O, e.g. CH_2, CCl_2, NH or CF_2). Preferably, a tri-n-butylammonium salt of the pyrophosphate or modified pyrophosphate in DMF is used. The resulting cyclic P(III)-P(V) intermediate (IIa) is then oxidized under anhydrous conditions, e.g. with a peroxide, such as t-butyl hydroperoxide, cumene hydroperoxide, (10-camphorsulfonyl)oxaziridine. Alternatively, phenylacetyldisulfide (V_2=S), or borane-diisopropylethylamine complex (V_2=BH_3) can also be employed respectively, to give the corresponding cyclic 5'-triphosphate/triphosphate analogue of formula (IIb). Reference in this context is also made to WO 96/40159 or WO 2009/060281.

[0069] Reaction step (a) may take place with an oligonucleotide in solution or with an oligonucleotide bound to a solid phase, e.g. an organic resin or glass, such as CPG. The oligonucleotide may further comprise protecting groups, e.g. sugar- or nucleobase protecting groups that are well known to the skilled person. Preferred examples of protecting groups are 2-cyanoethyl for the internucleoside phosphodiester or phosphorothioate, tert-butyldimethylsilyl, triisopropylsilyloxymethyl or bis(acetoxyethoxy)methyl for the ribose 2'-hydroxyl group, 4-t-butylphenoxyacetyl or phenoxyacetyl, acetyl, isobutyryl, benzoyl for the exocyclic amino groups of the nucleobases. More preferably, step (a) is carried out with a solid-phase bound oligonucleotide.

[0070] According to step (b) of the method of the disclosure, compound (IIb) is reacted with a capture tag agent of formula (III)

Z-Y-XH (III)

wherein X is a group selected from NH, NR^3, O or and X and Y are as defined above. R^3 is defined as described above for R^1.

[0071] In particular, in case for X being O an agent of limited nucleophilicity such as decanol can be used for ring opening (cf. Figure 1, step 4). Such a step can be carried out at room temperature, e.g. for 48 h, and allows conversion of the cyclotriphosphate to the desired triphosphate γ-ester.

[0072] According to step (c) the ON protection groups are cleaved off.

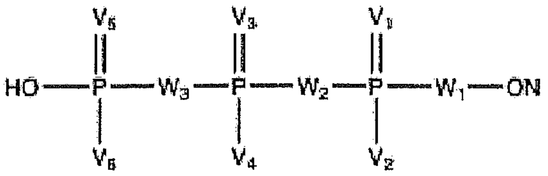
[0073] During step (c), for example, the deprotective reagents (a) and (b) specified above may be followed by deprotection condition with X = O over a period of 20 min to 180 min, more preferably 60 min to about 150 min, in particular about 120 min, at 60-70°C, in particular about 65°C. If X = NH, such reactions are excluded due to different binding stability or can only be carried out under significantly worse reaction parameters, e.g. over a period of 40 h at RT.

[0074] Step (d) of the disclosed method comprises contacting the reaction product of step (b), with a capture reagent capable of interacting with the capture tag Z under conditions which allow separation of the capture tag containing oligonucleotide (I) from other species contained in the reaction product. Before step (d), the solid phase bound oligonucleotide (I) is cleaved from the solid phase and deprotected, i.e. the protection groups are partially or completely removed. The capture reagent is preferably immobilized on a suitable support, e.g. a chromatographic support. In order to provide separation of capture tag containing oligonucleotide (I) from non-capture tag-containing species, the reaction products from step (b) are cleaved from a solid phase and deprotected, if necessary, and subjected to a separation procedure, preferably a chromatographic separation procedure based on the interaction of the capture tag Z with the capture reagent. During the separation step, the purity of the oligonucleotide (I), which is generally in the range of 25-70% for the crude material depending upon the length and complexity of the sequence, may be increased to 90%, 91%, 92%, 93%, 94%, 95% or more. For toxicity studies a purity of > 85% is desirable, whereas in late stage clinical trials the purity should be in the range of at least 90-95%. Thus, the disclosureprovides a way to obtain a high purity pppRNA as would be required for human clinical trials.

[0075] In step (d), the capture tag and the capture reagent capable of interacting therewith are preferably selected from (i) a hydrophobic or fluorinated group and a chromatographic material with affinity for hydrophobic or fluorinated groups, e.g. a reversed phase material or a fluorous affinity support; (ii) a first partner of a non-covalent high-affinity binding pair and a second complementary partner of a non-covalent high-affinity binding pair, (iii) a first partner of a covalent binding pair and a second complementary partner of a covalent binding pair, where the first and second partner form covalent bonds.

[0076] The capture tag is functionally defined below by a series of plausible Examples. A general rule may be: Z has to allow a convenient purification, and it should be removable under conditions which are compatible with pppRNA stability requirements.

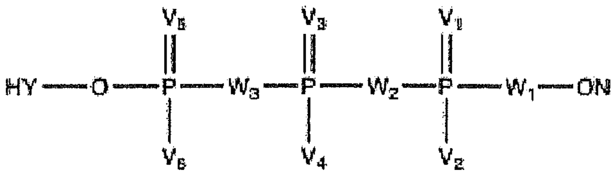
[0077] Additionally, the method may further comprises step (e) removing the capture tag to obtain an oligonucleotide of Formula (IV). According to a preferred instance of the disclosure, for X=O a compound of Formula (IV)a or (IV)b



$$HO-\underset{\underset{V_6}{|}}{\overset{\overset{V_5}{\|}}{P}}-W_3-\underset{\underset{V_4}{|}}{\overset{\overset{V_3}{\|}}{P}}-W_2-\underset{\underset{V_2}{|}}{\overset{\overset{V_1}{\|}}{P}}-W_1-ON$$

(IVa)

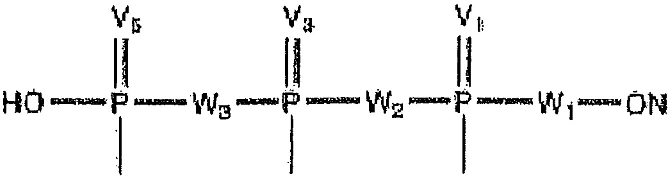
or



$$HY-O-\underset{\underset{V_6}{|}}{\overset{\overset{V_5}{\|}}{P}}-W_3-\underset{\underset{V_4}{|}}{\overset{\overset{V_3}{\|}}{P}}-W_2-\underset{\underset{V_2}{|}}{\overset{\overset{V_1}{\|}}{P}}-W_1-ON$$

(IVb)

and for X=NH a compound of Formula (IV)a



$$HO-\underset{\underset{V_6}{|}}{\overset{\overset{V_5}{\|}}{P}}-W_3-\underset{\underset{V_4}{|}}{\overset{\overset{V_3}{\|}}{P}}-W_2-\underset{\underset{V_2}{|}}{\overset{\overset{V_1}{\|}}{P}}-W_1-ON$$

(IVa)

is obtained.

[0078] Step (e) has to be compatible with stability requirements of the triphosphate end product and with stability requirements of the interribonucleotide bond. It may comprise cleavage by mildly acidic conditions when X is NH, cleavage with silver ions when X is S, cleavage by a thiol such as dithiothreitol leading to elimination of thiirane when Y-X-P contains -S-S-CH_2-CH_2-O-P.

[0079] In further instances of the disclosure the capture tag Z is not or not completely removed. In these instances the tagged oligonucleotide as such may have utility, e.g. utility as pharmaceutical agent.

[0080] In these instances, the reagent Z-Y-XH has to be selected from a subgroup of Z-residues, which are functionally compatible with the structural requirements of the RIG-I sensor. For instance, the Z=decyl-octadecyl, Y=link X=NH or O combination is known to fulfill these requirements.

[0081] The triphosphate/triphosphate analogue modified oligonucleotides produced according to the disclosure are particularly suitable for pharmaceutical applications due to their high purity. In an especially preferred instance of the disclosure, the oligonucleotide (I) or (IV) is an activator of RIG-I helicase. Specific examples of suitable RIG-I activators are disclosed in Schlee et al., 2009, supra.

Figure Legend

[0082]

Figure 1 shows a schematic overview of the synthesis method for oligonucleotide triphosphate ester derivatives.

Figure 2 shows RP-HPLC and ESI-LC/MS analysis of a decyl-O-pppRNA 24mer synthesis (RNA sequence: 5'-GACGCUGACCCUGAAGUUCAUCUU)

(A) RP-HPLC profiles of

a) crude reaction mixture containing 48% decyl-O-pppRNA (RT=14min)
b) pure decyl-O-pppRNA

Column: Hamilton PRP-1 4.1x250, 10μm
Gradient: 0-100% B in 18min, A=100mM TEAB; B=80% methanol, 100mM TEAB

(B) ESI-MS profile of pure decyl-O-pppRNA recorded using RP-LC/MS (MW calc.: 7987, found: 7968)

Figure 3 shows a semipreparative scale RP-HPLC purification of a 1μmol scale reaction of decyl-O-pppRNA: Decyl-tagging allows efficient separation of the desired product (fraction3) from non-tagged side products including synthesis failure sequences, full-length nonphosphorylated OH-RNA and nonderivatized pppRNA.

Column: Hamilton PRP-1 7x250mm, 10μm
Gradient: 0-80% B in 50min, A=100mM TEAB; B=80% methanol, 100mM TEAB.

Figure 4: Especially preferred instances of the disclosure of Y.

Figure 5: A screen of 2'-O-methylations resolves positions to introduce RIG-I selectivity.

Figure 6: Methylations render the duplex RIG-I specific and highly active.

Figure 7: Terminal phosphorothioates augment immunogenicity of the selective duplex.

Figure 8: Selected 2'-Fluoro-substitutions augment the RIG-I activation potential of the duplex.

Figure 9: The multiple modification pattern of OH ds-RNA OH-GFP2 leads to a strong enhancement of RIG-I activation potential.

Figure 10: Enhancement of immunogenicity by backbone modifications is transferable to a triphosphorylated duplex.

Figure 11: Shows a schematic overview of the method of the disclosure using diethylenglycolmonobutyletheras an example for alkylpolyethylenglycols as nucleophilic ring opening reagent.

Figure 12: RP- HPLC purification of C4-DEG-pppRNA crude reaction mixture containing 45 % C4-DEG-pppRNA (peak at 88.4 ml gradient volume) and 50 % pppRNA (peak at 77.5 ml) Column: Hamilton PRP-1 7 x 250 mm, 10 μm Flow rate 3ml/min.

Gradient: 1-80 % B in 50 min, A= 0.1M TEAB ; B= 80% Methanol 0.1 M TEAB

Figure 13: MALDI-TOF spectra corresponding C4-DEG-pppRNA after HPLC purification. The correct mass peak is observed at m/z 7972.6 (A).

Figure 14 shows the synthesis scheme of pppRNA using a lipophilic polyether composite tag.

Figure 15 shows the HPLC purification and MALDI data related to Fig 14 (RNA sequence: 5'-GACGCUGACCCUGAAGUUCAUCUU)

(A) RP-HPLC purification of

C11-CONH-$CH_2CH_2CH_2$-(O-CH_2CH_2)$_2$-O-$CH_2CH_2CH_2$-NHpppRNA
Column: Hamilton PRP-1 4.1x250mm, 10μm
Gradient: 0-80 %B in 50 min, ; A=100 mM TEAB, B=80 % methanol, 100mM TEAB

(B) MALDI spectrum recorded from the crude reaction mixture after desalting showing the presence of C11-CONH-$CH_2CH_2CH_2$-(O-CH_2CH_2)$_2$-O-$CH_2CH_2CH_2$NHpppRNA
(Mw 8214,8)
(C) MALDI spectrum of the pH=3.8 hydrolysis product of purified C11-CONH-$CH_2CH_2CH_2$-(O-CH_2CH_2)$_2$-O-$CH_2CH_2CH_2$NHpppRNA (Mw 7832.6)

Figure 16 shows the synthesis scheme of cholesteryl-tagged pppRNA with optional cleavage to the corresponding pppRNA.

Figure 17 shows the purification and analysis of a cholesteryl-tagged pppRNA (RNA sequence: 5'-GACGCUGACCCUGAAGUUCAUCUU):

(A) RP-HPLC purification of Cholesteryl -CONH-CH_2CH_2-(O-CH_2CH_2)$_2$-NH-pppRNA Column: Hamilton PRP-1 4.1x250mm, 10μm
Gradient: 0-10%B in 5min, 10%B for 9min, 10-100%B in 33min; A=50mM TEAB, B=95% methanol, 50mM TEAB
(B) RP-HPLC analysis of pure Cholesteryl -CONH-CH_2CH_2-(O-CH_2CH_2)$_2$-NH-pppRNA.

Column: Hamilton PRP-1 4.1x250mm, 10μm
Gradient: 0-100%B in 18min, 100%B for 4min; A=50mM TEAB, B=95% methanol, 50mM TEAB

(C) MALDI spectrum of pure Cholesteryl -CONH-CH_2CH_2-(O-CH_2CH_2)$_2$-NH-pppRNA.
The pppRNA peak (7827.3Da) is due to PN-cleavage during the ionisation process.

[0083] The invention is defined in the appended claims. Any example lying outside the scope of said claims is only intended for illustrative as well as comparative purposes.

Example 1: Preparation of 5'-Decyl-O-triphosphate RNA

[0084] As outlined in the overview (Figure 1) the decyl-O-triphosphate RNA synthesis process includes the following synthesis steps:

1-4) 5'-Decyl-O-triphosphate RNA

[0085] The support-bound, fully protected 5'OH-RNA (1μmol) was dried for 3h under vacuum within a synthesis column and subsequently washed with anhydrous pyridine/dioxane (1:3, v/v, 4ml). Under argon, a freshly prepared 1M solution of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in dry dioxane (100μl, 100μmol) was injected into a flask containing 2ml of anhydrous pyridine/dioxane (1:3, v/v). The resulting 50mM phosphitylating solution was drawn into the synthesis column and slowly moved back and forth during a reaction time of 30min. Next, a tetra(tri-n-butylammonium) pyrophosphate solution was prepared by mixing a 0,5M solution of bis(tri-n-butylammonium) pyrophosphate in DMF (1ml, 0,5mmol) and tri-n-butylamine (238μl, 1mmol). The pyrophosphate solution was pushed through the column and after expelling and discarding the excess phosphitylating reagent from the column the remaining pyrophosphate solution was pushed back and forth using two syringes. After 10min the column was washed with anhydrous acetonitrile (3ml). A 5,5M tert-butyl hydroperoxide solution in decane (300μl) was dissolved in anhydrous acetonitrile (2ml) and brought into contact with the synthesis support. After 15 min the column was washed with anhydrous acetonitrile (6ml). Subsequently, a homogeneous solution of N-methylimidazole (240μl, 3mmol), tri-n-butylamine (250μl, 1,1mmol) and n-decyl alcohol (2ml, 10,5mmol) was repeatedly pushed to and fro through the column and was left to react for 48h for cyclotriphosphate conversion to decyl-O-triphosphate. The column was washed with anhydrous acetonitrile (6ml) and treated with 0,1M triethylammonium bicarbonate (TEAB, 2ml) for 20min for hydrolysis of unreacted cyclotriphosphate, avoiding unspecific derivatisation during the following deprotection procedure. Following a further wash step with anhydrous acetonitrile

(9ml) the synthesis support was dried in a stream of argon.

5-6) Deprotection & purification

[0086] The 5'-decyl-O-triphosphate oligonucleotide was brought into contact with a freshly prepared solution of 40% aqueous methylamine and concentrated aqueous ammonia (AMA, 1:1, v/v, 2ml) using two syringes. After 30min cleavage time the solution was transferred to a clean screw cap vial and the support was rinsed with AMA (1ml). The combined solution and washing was heated for 10min at 65°C. After cooling on ice the solution was evaporated to dryness and the residue was dried by co-evaporation with absolute ethanol. For removal of the 2'-O-TBDMS protecting groups treatment with triethylamine trihydrofluoride (TEA.3HF) can be used without significant loss of the modified triphosphate moiety. The decyl-O-triphosphate oligonucleotide was redissolved in a freshly prepared solution of N-methylpyrrolidone/triethylamine/TEA.3HF (NMP/TEA/TEA.3HF, 6:4:3, v/v, 325μl) and the solution was heated at 65°C for 2h. Alternatively, a deprotection solution of TEA.3HF in DMSO (1:1, v/v, 600μl) could be used. The fully deprotected decyl-O-triphosphate oligonucleotide was precipitated from the deprotection solution using n-butanol and purified by HPLC. The lipophilic decyl-tag allows separation of the decyl-O-triphosphate from impurities that do not contain the tag by using reversed phase chromatography. The reaction product was applied to a 7x250mm PRP-1 column and separated in a linear gradient from 0 to 100% buffer B in 50 min at a flow rate of 3ml/min. Buffer A was 100mM TEAB and buffer B 100mM TEAB in 80% methanol. The product fractions were collected, evaporated and desalted by repeated co-evaporation with methanol. The residue was dissolved in water and transferred to the sodium form by ethanol precipitation in the presence of 0,3 M sodium chloride.

Example 2

Preparation of a 5'-pppRNA gamma 2-(2-Butoxyethoxy)ethyl-ester (C4-DEG-pppRNA)

[0087] An overview of the reaction scheme described in Example 2 is shown in Fig. 11.

[0088] **Step 1:** Dissolve 203 mg (1 mmol) of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one in 1 mL of dry dioxane in a 10 mL septum vial under argon.

[0089] **Step 2:** Dry the synthesis column containing the fully protected RNA that has been detitrylated and thoroughly washed with acetonitrile, in vacuum for 12 h. Wash the column contents thoroughly by repeatedly drawing in and expelling 2 mL of anhydrous dioxane/pyridine solution, 3:1 (v/v) in an argon atmosphere.

[0090] **Step 3:** Add into a vial first 2 mL of pyridine/dioxane, 3:1 v/v followed by 100 μL of 1 M 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one solution in dry dioxane to give a 50 mM solution of the phosphitylating reagent, e.g. 2-chloro-4H-1,3,2-benzodioxaphosphorin-2-one, in dioxane/pyridine, 3:1 (v/v). Homogenize the solution by gently shaking. Start the reaction by drawing the 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one solution through the synthesis column from the vial.

[0091] During the reaction, repeatedly draw in and expel the 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one containing solution from the synthesis column, in order to allow thorough contact and good mixing with the solid phase supported RNA. A 30 min reaction time usually gives near quantitative reaction of the free 5'-OH group of the support bound oligomer in the 20-40 nt range.

[0092] **Step 4:** After a 30 min reaction time expel the dioxane/pyridine solution containing the excess phosphitylating agent into a waste container, fill a new syringe with a vortexed mixture of 1 mL of 0.5 M (Bu3NH)2 pyrophosphate in dry DMF and 238 μL (1 mmol) of dry Bu3N to give a 0.5 M (Bu3N)4 pyrophosphate solution. Push this solution through the column thereby replacing the dioxane/pyridine solution. The large excess of the pyrophosphate ensures a quantitative conversion of the intermediate to the P(III)-P(V) cyclic anhydride IIa.

[0093] **Step 5:** Wash the column with 3 mL of CH3CN to remove the DMF and excess PPi, and to fill the column reactor with dry CH3CN.

[0094] **Step 6:** Dissolve 300 μL of t-BuOOH (5.5 M solution in decane, Sigma-Aldrich) in 2 mL of anhydrous CH3CN to give an approximately 0.7 M homogeneous solution. Contact the synthesis support with this solution for 15 min in order to obtain the oxidized P(V) cyclic anhydride IIb.

[0095] **Step 7:** Wash the column with 3 mL of dry CH3CN to remove the excess peroxide and fill it with dry CH3CN.

[0096] **Step 8:** Contact 2ml dry 2-(2-Butoxyethoxy)ethanol (Diethylene glycol monobutyl ether) containing 0.1 M N-methylimidazole and 0.1 M tri-n-butylamine with the support in the column. Contact time of the CPG with the alcohol should be at least 48 hrs at room temperature.

[0097] **Step 9:** Wash the column thoroughly with 9 mL acetonitrile, then contact the column with 2ml 0.1 M TEAB (triethylammonium bicarbonate), solution in water for 1 hr in order to hydrolyse unreacted cyclotriphosphate.

[0098] **Step 10** - **First stage of the deprotection:** Pass 1 mL of deprotection solution (40% aq. methylamine/conc. aq. ammonia 1:1 v/v. AMA reagent) through the support for 2-3 times. After a contact of 30 min transfer the solution into a new vial. Wash the support with same volume of AMA deprotection solution and combine the washings. Heat the combined solution and washings for 10 min at 65°C. After cooling on ice, concentrate the solution to a volume of 300-500 μL, then evaporate to dryness.

[0099] **Step 11** - **Removal of the 2'-O-TBDMS protecting groups:** Dry the residue by addition and coevaporation of 300 μL of dry EtOH, add 1 mL of dry 1 M TBAF (tetra-n-butylammonium fluoride) in THF, seal tightly and put on a shaker for 16 h. Quench the reaction with 1 mL of sterile aqueous 1 M TEAB (triethylammonium bicarbonate), and desalt it on a NAPTM-25 (Nucleic Acid Purification) column using sterile water as eluent. Filtration through a sterile 2 μm filter may be necessary at this step. Combine and evaporate the UV-absorbing fractions to a volume of 150 μL, add 100 mL of 1 M TEAB pH8 and store the solution frozen at -20°C until the HPLC purification can be performed.

[0100] **Step 12** - **HPLC purification:** The reaction product from an 1 μmol scale reaction mixture from step 11 was loaded into a 7x25 mm PRP-1 column (Hamilton). Purification was performed using a linear gradient buffer B from 0 to 80% in 50 min at a flow rate of 3 mL/min. Buffer A is 100 mM TEAB and buffer B is 100 mM TEAB in methanol/water 8:2 v/v. A typical example of a 24-mer purification is shown in Figure 3. A combination of a 4 atom alkyl tag and diethyleneglycol residue is sufficient for near base line separation from pppRNA. Fraction corresponding to the peak at 88.4 ml were combined, evaporated on a rotary evaporator and desalted by several coevaporations with dry methanol.

Example 3

Derivatisation of pppRNA using lipophilic polyether composite tags with preformed lipophilic polyether amines

[0101] **Example 3** employs lipophilic polyether amines of the structure ZY-H to generate ZYNHpppRNA

Part A: Economical large scale preparation of the lipophilic polyether amine

[0102] This procedure can be performed without flash column chromatography and is generally applicable for the synthesis of monoamides from water-soluble diamines and methyl esters of lipophilic carboxylic acids.

[0103] Synthesis of N-Lauryl-4,7,10-trioxa-1,13-tridecanediamine (compound 3, Fig. 14): 4,7,10-trioxa-1,13-tridecanediamine (43,8 ml, 200 mmol) was dissolved in 32.5 ml Methanol. Lauric acid methyl ester (4.92 ml, 20 mmol) was added slowly with stirring and the closed flask was kept at room temperature for 5 days. Methanol was removed from the reaction mixture on a rotary evaporator and the residue was dissolved in 100 ml Ethylacetate.

[0104] The excess of the diamine was removed by extraction with water (2*120 ml) followed by concentrated sodium chloride (2*100 ml). The ethylacetate phase was evaporated and the residual oil was crystallized upon standing at -20 °C to give 6.8 g (17 mmol) of pure N-Lauryl 4,7,10-trioxa-1,13-tridecanediamine.

Part B: Ring opening reactions on the CPG bound RNA cyclotriphosphate with N-Lauryl-4,7,10-trioxa-1,13-tridecanediamine

[0105] CPG bound RNA cyclotriphosphate (compound 4, Fig. 14) was synthesized according to steps 1-7 in the previous example 2. The ring opening reaction of solid phase immobilized RNA cyclotriphosphate was performed with 0,08 M solution of N-Lauryl-4,7,10-trioxa-1,13-tridecanediamine in acetonitrile. The acetonitrile solution was contacted with the CPG for 3 hrs a at room temperature, the support was then washed with 10 ml dry acetonitrile and flushed dry with argon. Cleavage from the support, deprotection and further HPLC processing was performed exactly according to steps 10-12 of example 2. Product 5 was eluted at 75% Methanol concentration (Fig. 15A). MALDI spectral analysis of the reaction mixture confirms the structure of compound **5** (Mw 8214.8Da, Fig. 15B).

[0106] Optionally, the purification tag may be removed to obtain the corresponding triphosphate oligonucleotide: 100 nmol n-lauryl subtituted gamma amid (compound 5, Fig. 14) was dissolved in 400 μl of pH 3.8 deprotection buffer in a 2 ml Eppendorf tube and the sealed tube was heated at 60° C for 70 min. These conditions result in quantitative cleavage of the phosphoramidate bond of compound **5**, with no degradation of the triphosphate moiety. The reaction mixture was cooled on ice and 14 μL of sterile 5M NaCl solution and 1.2 mL of absolute ethanol was added. The precipitate was collected by centrifugation, washed with cold ethanol, dried on a Speed Vac, dissolved in sterile water and stored frozen at -20 °C.

[0107] MALDI spectral analysis confirms the expected MW of the pppRNA product (Mw 7832 Da, Fig 15C).

Example 4:

On-column approach for the derivatisation of pppRNA with lipophilic polyether composite tags

[0108] **Example 4** is a two-step procedure employing on-column derivatisation of an immobilized HYNHpppRNA oligonucleotide to generate ZYNHpppRNA

Preparation of Cholesteryl-tagged triphosphate RNA (Chl-CONH-CH_2CH_2-(O-$CH_2CH_2)_2$-NH-ppp-RNA, compound 4, Fig. 16)

[0109] Conjugation of a lipophilic cholesteryl-tag to triphosphate RNA allows efficient separation of the tag-pppRNA product from non-tagged impurities by RP-HPLC. An overview of the reaction scheme for preparation of cholesteryl-tagged triphosphate RNA is shown in Fig. 16. In a first step the support-bound, fully protected 5'OH-RNA was converted into the cyclic triphosphate intermediate (compound **2**, Fig. 16) as described in steps 1-7 of example 2.

[0110] Subsequently, the column was washed with anhydrous acetonitrile (3 ml). 176 μl of 2,2'-(ethylenedioxy)diethylamine (compound **1**, Fig. 16; 1,2 mmol) were dissolved in anhydrous acetonitrile (1 ml) and the solution was brought into contact with the support in the column. After 3 min the column was washed with anhydrous acetonitrile (3 ml) and anhydrous dichloromethane (6 ml).

[0111] Subsequently, the amino-modified triphosphate (i.e. compound **3**, Fig. 16) was treated with a premixed solution of cholesteryl chloroformate (36 mg, 80 μmol), N,N-diisopropylethylamine (13,9 μl, 40 μmol) and 4-dimethylaminopyridine (4,9 mg, 40 μmol) in anhydrous dichloromethane (5 ml) for 15 min. The column was washed with anhydrous dichloromethane (3ml) and anhydrous acetonitrile (6ml) and dried in a stream of argon. For cleavage from the support and deprotection, the derivatized oligonucleotide was brought into contact with a freshly prepared solution of 40% aqueous methylamine and concentrated aqueous ammonia (AMA, 1:1, v/v, 2 ml) using two syringes. After 30 min cleavage time the solution was transferred to a screw cap vial, heated for 10 min at 65 °C and evaporated to dryness. The residue was dried by co-evaporation with absolute ethanol and treated with 1M tetra-n-butylammonium fluoride in THF (1 ml, 1 mmol) with shaking for 16 h. After desalting on a NAP-25 column the fully deprotected cholesteryl-tagged triphosphate oligonucleotide was purified by HPLC using a reverse phase column (Hamilton PRP-1, 4.1x250 mm, Fig. 17A). Compound **4** eluted at 95% methanol concentration and the expected structure was confirmed by MALDI analysis (Mw 8370,6 Da, Fig. 17C).

[0112] Optionally, the cholesteryl-tag is removed by acid hydrolysis at pH 3.8 at 60 ° C to obtain the corresponding triphosphate oligonucleotide (compound **5**, Fig. 16) following the same procedure as described in example 3.

Example 5: The systematic introduction of 2'-O-methylations results in a RIG-I selective RNA duplex

[0113] A list of abbreviations and their meaning may be found at the end of the application text.

[0114] A body's own RNA is characterised by a plurality of modifications that may have an influence on RNA functions (tRNAs and rRNAs). 2'-O-methylations are thus able to modify the immunogenicity of the RNA. A selective RIG-I ligand should therefore not comprise more than one 2'-O-methylation per strand. These properties are included in the RNA duplex by combination of suitably methylated sense and antisense strands.

[0115] A screen of 2'-O-methylations resolved positions to introduce RIG-I selectivity (Fig. 5). For the sense (A) and antisense (B) strand of OH-GFP2 a full 2'-O-methylation screen was performed. Each position was modified individually by 2'-O-methylation and used for stimulation of RIG-I, TLR7 or TLR8. For stimulation of RIG-I, peripheral blood mononuclear cells (PBMCs) were blocked with Chloroquine and stimulated by lipofection of the duplex. IFNa was measured 20h post stimulation by ELISA. For TLR7 and TLR8 only the relevant single-strand was screened for stimulatory capacity and checked on unblocked PBMCs. The single-strands were transfected by complexation with poly-L-Arginine and 20h post transfection for TLR7 activation IFNa, for TLR8 activation IL12p70 was measured by ELISA. 100% induction with GFP2 for the sense strand corresponds to 3085 pg/ml IFNa for RIG-I, 2758 pg/ml IFNa for TLR7 and 1206 pg/ml IL12p70 for TLR8. For the antisense 100% of GFP2 corresponds to 2342 pg/ml IFNa for RIG-I, 1831 pg/ml IFNa for TLR7 and 3018 pg/ml IL12p70 for TLR8. An overview of the impact of position dependent introduction of 2'-O-methylation is given in table form (Figure 5). Effects are normalized to the unmodified strand as a reference. White = no change, yellow = reduction of immune stimulation, red = no immune stimulation, green = augmented immune stimulation.

[0116] Furthermore, it could be shown that certain methylations render the duplex RIG-I specific and highly active.

[0117] Fig. 6: (A) Methylations inert for RIG-I activation were combined in the antisense strand and the resulting duplex was used for stimulation of chloroquine-blocked PBMCs at a concentration of 0,8 μg/ml. IFNa was measured by ELISA at 20h post stimulation. 100% of OH-GFP2 resembles 2729 pg/ml IFNa. (B) Different combinations of 2'-O-methylations in sense and antisense strand were hybridized and used for stimulation of PBMCs. For the stimulation of RIG-I they were blocked with Chloroquine and transfected with Lipofectamine, for TLR7 and TLR8 unblocked PBMCs were used and transfected with duplexes complexed with poly-L-Arginine. 100% for IVT-2 correspond to 4861 pg/ml IFNa for RIG-I 100% at 9.2S correspond to 1975 pg/ml IFNa for TLR7 and 771 pg/ml IL12p70 for TLR8. (A) and (B) show mean ± SEM of 3 donors.

Example 6: Increase of RIG-I activation by insertion of RNA-stabilising modifications

[0118] During siRNA therapy short dsRNA fragments are inserted in a body, where they inhibit the formation of a specific protein in the target cells. The problem with this form of therapy is the enormous instability of the siRNA duplexes. RNA may easily be degraded by exonucleases and endonucleases in the serum during transport to the target cell.

[0119] It seems probable that a significant part of exogenous RNA used for therapeutic purposes is degenerated in serum and cytosol of the subject to which it is administered.

[0120] It could be shown that each 5'-PTO modification has a positive effect on duplex immune activity and that the combination of 5'PTO in the sense and antisense strand leads to a maximum increase in activity (s15/as3 against s15 PTO/as3 PTO; Figure 7 B). The use such modifications of the RNA backbone in RIG-I selective ligands can thus be regarded as beneficial.

[0121] In particular, it could be shown that 5'-phosphorothioates augment immunogenicity of a selective duplex.

[0122] Fig. 7: (A) PBMCs were blocked with Chloroquine and stimulated with duplexes at a concentration of 0,8 μg/ml. 20h post stimulation IFNa was measured by ELISA. 100% of IVT2 corresponds to 6138 pg/ml IFNa. (B) Indicated duplexes were titrated and used for stimulation of RIG-I. PBMCs were blocked with Chloroquine and duplexes were transfected using Lipofectamine. 20h post stimulation IFNa was determined in the supernatant by ELISA. 100% of 50nM 3P-GFP2 corresponds to 16044 pg/ml IFNa. Results show mean ± SEM of 4 donors.

[0123] In the development on therapeutic siRNAs the problem of serum stability was solved by another kind of modification: if the 2'-fluoro substitutions were incorporated in the RNA, the resulting RNAs were more stable with regard to nuclease digestion. However, it was observed that in case of RIG-I dependent immune stimulation a 2'-fluoro substitution could activate immune stimulation.

[0124] In particular, it could be shown that 5'-phosphorothioates augment immunogenicity of a selective duplex.

[0125] However, according to the present invention it was found that selected 2'-fluoro substitutions augment the RIG-I activation potential of the duplex.

[0126] For the sense (A) and antisense (B) strand of the duplex each position was modified by a 2'-Fluoro-substitution individually (Fig. 8). Resulting duplexes were used for stimulation of PBMCs at a concentration of 0,8 μg/ml. For the assessment of RIG-I activation PBMCs were blocked and transfected by Lipofectamine, for stimulation of TLR7 and TLR8 PBMCs were used unblocked and transfected with duplexes complexed with poly-L-Arginine. 20h post stimulation cytokines was measured by ELISA. 100% for GFP2 in the sense strand resembles 2407 pg/ml IFNa for RIG-I, 3281 pg/ml for TLR7 and 1990 pg/ml for TLR8. For the antisense strand 100% GFP2 corresponds to 4512 pg/ml IFNa for RIG-I, 4691 pg/ml IFNa for TLR7 and 1997 pg/ml IL12p70 for TLR8. Shown is mean ± SEM of 4 donors.

[0127] Thus, it was very important to establish a duplex as active as possible which can compensate the loss of RNA by its strong activity. In the previous passage it was described how RNA modifications could be identified by selective PTO bindings and 2'-fluoro substitutions, which led to an increase in the activity of a duplex.

Example 7: The combination of all modifications leads to a strongly immunogenic RIG-I ligand

[0128] In order to test if PTO bindings or 2'-fluoro substitutions can be combined with 2'-O-methylations to increase selectivity, they were combined in a duplex step-by-step.

[0129] PBMCs were blocked with Chloroquine and used for stimulation of RIG-I. Differentially modified duplexes based on OH-GFP2 were used and RIG-I activation was measured at 20h post stimulation by ELISA on IFNa (Fig. 9). Multiply modified duplexes were either compared to OH-GFP2 (A) or triphosphorylated 3P-GFP2 (B). 100% of IVT 4 corresponds to 21305 pg/ml of INFa. Shown is mean ± SEM of 2 donors.

[0130] The 2'-O methylations s15/as3 were added to the starting RNA OH-GFP2 first (OH-GFP2 oMet15s/oMet3as, Figure 9 A) and then compared to the 5'-PTO modification in *sense* and *antisense* (OH-GFP2 PTO 5', Figure 9 A). Subsequently, both types of modification were combined with a duplex (OH-GFP2oMet15/oMet3 PTO, Figure 9 A). Finally, a duplex was generated that additionally comprised three 2'-fluoro substitutions at positions 7 & 23 in *sense* and at position 13 in *antisense* (OH-GFP2oMet15/3-F23/13-PTO, Figure 9 A). The comparative titration of these RNA double-strands showed that the multi-modified oligo OH-GFP2oMet15/3-F23/13-PTO surpassed other duplexes in activity by far. To better evaluate its activity, the duplex was titred and compared to its unmodified triphosphate duplex (Figure 9 B). It was found that the insertion of a modification made it possible to change the oligonucleotide so that its immune activity compared to that of its triphosphate equivalent (3P-GFP2, Figure 9 B).

Example 8: The elements are transferable to the 3P-dsRNA system

[0131] The whole development of the RIG-I selective ligand was carried out on the OH level of the sequence GFP2. Although the activity could be increased immensely by suitable modifications, a triphosphorylation of a ligand would increase its activity additionally.

[0132] So far it was not clear whether the modifications and their respective positioning could just be transferred to the 3P-GFP2 system.

[0133] In order to find an answer to this question duplexes were produced, which all had the modification that increased the activity: 2'-O methylation at base 15 in the *sense* strand, 2'-fluoro substitution at base 7 and 23 and two PTO bindings at the 5' and the 3' end (2S2F, Figure 10). In the *antisense* strand a 2'-O methylation at base 3, a 2'-fluoro substitution at base 13 and two 5'-PTO binding were combined. To avoid that the two 5'-PTO bindings at the *sense* strand intermit by their proximity to the triphosphate, a multi-modified sense strand without 5'-PTOs was produced additionally (1S2F, Figure 10).

[0134] In order to be able to estimate the gain in activity by triphosphorylation, the duplexes were each produced with 5'-hydroxyl and 5'-triphosphate (OH vs 3P, Figure 10) and compared to each other by stimulation titration on PBMCs.

[0135] Fig. 10: (A) Multimodified duplexes were synthesized with and without triphosphate. A dose titration was performed on Chloroquine blocked PBMCs. 20h post stimulation immune activation was measured by ELISA on IFNa. Shown is mean ± SEM of 4 donors. (B) Based on the titration curves the biological EC50 values were calculated.

[0136] It was found that the multi-modified OH duplexes, irrespective of the presence of one or two PTO ends, could reach the activity limits of the unmodified form of OH-GFP2 (OH Multi 1S2F, OH Multi 2S2F, OH-GFP2, Figure 10). The additional addition of 5'-triphosphate at the *sense* strand resulted in an additional increase in activity by factor 5 compared to the hydroxyl complexes (3P-Multi 1S2F, 3P-Multi 2S2F, Figure 10).

[0137] As a result it was observed that the activity-increasing effects of various modifications can be combined with the triphosphorylation of the oligonucleotide and that they also show their positive influence there.

[0138] By the progressive increase of the original duplex OH-GFP2 on the level of selectivity (2'-O methylation), the increase in activity by stabilising modifications (PTO bindings and 2'-fluoro substitutions) and the affinity to the binding pocket (triphosphate), it became possible to define a potent, selective RIG-I ligand (3P-Multi 1S2F).

[0139] Finally, a 3P-dsRNA could be developed in which the immune-stimulating activity was increased to a maximum by the specific insertion of modifications. This increase in immunogenicity makes it possible to keep the dosage of a medicament on a low level, and to compensate losses in RNA that occur in the period from the application of a medicament until the entry into a cell.

GFP2s oMet1	G_mACGCUGACCCUGAAGUUCAUCUU C UGCGACUGGGACUUCAAGUAGAA	N_m= 2'-OCH_3
.		
GFP2s oMet2	GA$_m$CGCUGACCCUGAAGUUCAUCUU CU GCGACUGGGACUUCAAGUAGAA	
.		
.GFP2s oMet23	GACGCUGACCCUGAAGUUCAUCU_mU CUGCGACUGGGACUUCAAGUAGA A	
.		
GFP2s oMet24	GACGCUGACCCUGAAGUUCAUCUU_m CUGCGACUGGGACUUCAAGUAGAA	
GFP2as oMet1	G ACGCUGACCCUGAAGUUCAUCUU C_mUGCGACUGGGACUUCAAGUAGAA	N_m= 2'-OCH_3
.		
GFP2as oMet2	GA CGCUGACCCUGAAGUUCAUCUU CU_mGCGACUGGGACUUCAAGUAGAA	
.		
.		
GFP2as oMet23	GACGCUGACCCUGAAGUUCAUCU U CUGCGACUGGGACUUCAAGUAGA_mA	
.		
GFP2as oMet24	GACGCUGACCCUGAAGUUCAUCUU CUGCGACUGGGACUUCAAGUAGAA_m	
OH-GFP2 multi oMet	G AC G CUGAC C C U GAAGUUCA UCUU C_mU$G_m$$C_m$GACU$G_m$$G_m$$G_m$$A_m$CUUCAAG$U_mAGAA_m$	N_m= 2'-OCH_3
s20/as12	GACGCUGACCCU GAAGUUCA_mUCUU CUGCGACUGGGA_mCUUCAAGU AGAA	N_m= 2'-OCH_3
s20/as19	GACGCUGACCCUGAAGUUC A_mUCUU CUGCGACUGGGACUUCAAG_mU AGAA	N_m= 2'-OCH_3
s15/as12	GACGCUGACCCU GAA_mGUUCAUCUU CUGCGACUGGGA_mCUU CAAGUAGAA	N_m= 2'-OCH_3
s15/as19	GACGCUGACCCUGAA_mGUUC AUCUU CUGCGACUGGGACUU CAAG_mUAGAA	N_m= 2'-OCH_3
s20/as3	GAC GCUGACCCUGAAGUUCA_mUCUU CUG_mCGACUGGGACUUCAAGU AGAA	N_m= 2'-OCH_3

s15/as3	GAC GCUGACCCUGAA$_m$GUUCAUCUU CUG$_m$CGACUGGGACUU CAAGUAGAA	N$_m$= 2'-OCH$_3$
s3/as12	GAC$_m$GCUGACCCU GAAGUUCAUCUU CUG CGACUGGGA$_m$CUUCAAGUAGAA	N$_m$= 2'-OCH$_3$
s3/as19	GAC$_m$GCUGACCCUGAAGUUC AUCUU CUG CGACUGGGACUUCAAG$_M$UAGAA	N$_m$= 2'-OCH$_3$
Fs1	G$_F$ ACGCUGACCCUGAAGUUCAUCUU C UGCGACUGGGACUUCAAGUAGAA	N$_F$ = 2'-F
.		
Fs2	GA$_F$ CGCUGACCCUGAAGUUCAUCUU CU GCGACUGGGACUUCAAGUAGAA	
.		
Fs23	GACGCUGACCCUGAAGUUCAUCU$_F$ U CUGCGACUGGGACUUCAAGUAGA A	
.		
Fs24	GACGCUGACCCUGAAGUUCAUCUU$_F$ CUGCGACUGGGACUUCAAGUAGAA	
Fas1	G ACGCUGACCCUGAAGUUCAUCUU C$_F$ UGCGACUGGGACUUCAAGUAGAA	N$_F$ = 2'-F
.		
Fas2	GA CGCUGACCCUGAAGUUCAUCUU CU$_F$ GCGACUGGGACUUCAAGUAGAA	
.		
Fas23	GACGCUGACCCUGAAGUUCAUCU U CUGCGACUGGGACUUCAAGUAGA$_F$ A	
.		
Fas24	GACGCUGACCCUGAAGUUCAUCUU CUGCGACUGGGACUUCAAGUAGAA$_F$	

3P-GFP2	ppp-GACGCUGACCCUGAAGUUCAUCUU CUGCGACUGGGACUUCAAGUAGAA	
s15/as3 PTO	GAC GCUGACCCUGAA$_m$GUUCAUC U U CUG$_m$CGACUGGGACUU CAAGUAG*A*A	* = PTO N_m= 2’-OCH$_3$
s15 PTO/as3	G*A*C GCUGACCCUGAA$_m$GUUCAUCUU C U G$_m$CGACUGGGACUU CAAGUAGAA	* = PTO N_m= 2’-OCH$_3$
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OH-GFP2 oMet15/oMet3 PTO	G*A*C GCUGACCCUGAA$_m$GUUCAUC U U C U G$_m$CGACUGGGACUU CAAGUAG*A*A	* = PTO N_m= 2’-OCH$_3$
OH-GFP2 F23s/13as	GACGCUGACCCUG AAGUUCAUCU$_F$ U CUGCGACUGGGAC$_F$UUCAAGUAGA A	N_F = 2’-F
OH-GFP2oMet15/3 F23/13 PTO	G*A*C GCUGACCCUG AA$_m$GUUCAUC U$_F$ U C U G$_m$CGACUGGGAC$_F$UU CAAGUAG*A*A	N_F = 2’-F * = PTO N_m= 2’-OC H$_3$
3P-GFP2 1S2F	PPP-GAC GCUG$_F$ACCCUG AA$_m$GUUCAUC*U$_F$*U CUG$_m$CGAC UGGGAC$_F$UU CAAGUAG*A*A	N_F = 2’-F * = PTO N_m= 2’-OC H$_3$
OH-GFP2 1S2F	GAC GCUG$_F$ACCCUG AA$_m$GUUCAUC*U$_F$*U CUG$_m$CGAC UGGGAC$_F$UU CAAGUAG*A*A	N_F = 2’-F * = PTO N_m= 2’-OC H$_3$
3P-GFP2 2S2F	PPP-G*A*C GCUG$_F$ACCCUG AA$_m$GUUCAUC*U$_F$*U C U G$_m$CGAC UGGGAC$_F$UU CAAGUAG*A*A	N_F = 2’-F * = PTO N_m= 2’-OC H$_3$
OH-GFP2 2S2F	G*A*C GCUG$_F$ACCCUG AA$_m$GUUCAUC*U$_F$*U C U G$_m$ CGAC UGGGAC$_F$UU CAAGUAG*A*A	N_F = 2’-F * = PTO N_m= 2’-OC H$_3$

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Claims

1. An oligonucleotide comprising a sense strand and an antisense strand, wherein the oligonucleotide comprises

   a sense strand comprising nucleotide sequence
   5' GACGCUGACCCUGAA$_{m}$GUUCAUCUU 3'; and
   an antisense strand comprising nucleotide sequence
   3' CUGmCGACUGGGACUUCAAGUAGAA 5';

   wherein the nucleotide indexed m is 2'-O-methylated.

2. The oligonucleotide of claim 1, wherein the sense strand and/or the antisense strand further comprises a phosphothioate bond, and wherein the oligonucleotide comprises

   a sense strand comprising nucleotide sequence

5’ GACGCUGACCCUGAA$_{m}$GUUCAUCUU 3’, and
an antisense strand comprising nucleotide sequence
3’ CUG$_{m}$CGACUGGGACUUCAAGUAG$_{PTO}$A$_{PTO}$A 5’; or
a sense strand comprising nucleotide sequence
5’ G$_{PTO}$A$_{PTO}$CGCUGACCCUGAA$_{m}$GUUCAUCUU 3’, and
an antisense strand comprising nucleotide sequence
3’ CUGmCGACUGGGACUUCAAGUAGAA 5’; or
a sense strand comprising nucleotide sequence
5’ G$_{PTO}$A$_{PTO}$CGCUGACCCUGAA$_{m}$GUUCAUCUU 3’, and
an antisense strand comprising nucleotide sequence
3’ CUG$_{m}$CGACUGGGACUUCAAGUAG$_{PTO}$A$_{PTO}$A 5’;

wherein

the nucleotide indexed m is 2’-O-methylated; and
the index PTO between two nucleotides indicates that said two nucleotides are linked by a phosphothioate bond.

3. The oligonucleotide of claim 1, wherein the sense strand and the antisense strand further comprises a phosphothioate bond and a 2’-fluoro modification, and wherein the oligonucleotide comprises

a sense strand comprising nucleotide sequence
5’ G$_{PTO}$A$_{PTO}$CGCUG$_{f}$ACCCUGAA$_{m}$GUUCAUCU$_{f}$U 3’, and
an antisense strand comprising nucleotide sequence
3’ CUG$_{m}$CGACUGGGAC$_{f}$UUCAAGUAG$_{PTO}$A$_{PTO}$A 5’; or
a sense strand comprising nucleotide sequence
5’ G$_{PTO}$A$_{PTO}$CGCUG$_{f}$ACCCUGAA$_{m}$GUUCAUC$_{PTO}$U$_{fPTO}$U 3’, and
an antisense strand comprising nucleotide sequence
3’ CUG$_{m}$CGACUGGGAC$_{f}$UUCAAGUAG$_{PTO}$A$_{PTO}$A 5’; or
a sense strand comprising nucleotide sequence
5’ GACGCUG$_{f}$ACCCUGAA$_{m}$GUUCAUC$_{PTO}$U$_{fPTO}$U 3’, and
an antisense strand comprising nucleotide sequence
3’ CUG$_{m}$CGACUGGGAC$_{f}$UUCAAGUAG$_{PTO}$A$_{PTO}$A 5’;

wherein

the nucleotide indexed m is 2’-O-methylated;
the nucleotide indexed f is 2’-fluoro; and
the index PTO between two nucleotides indicates that said two nucleotides are linked by a phosphothioate bond.

4. The oligonucleotide of any of claims 1 to 3, wherein the oligonucleotide is a RIG-I ligand.

5. A pharmaceutical composition comprising an oligonucleotide according to any of claims 1 to 3.

6. The pharmaceutical composition of claim 5, wherein the pharmaceutical composition is to be administered systemically or locally.

7. The pharmaceutical composition of claim 6, wherein the pharmaceutical composition is to be administered intraperitoneally, intramuscularly, intravenously, intranasally, subcutaneously, intradermally, or intrathecally.

8. The pharmaceutical composition of claim 7, wherein the pharmaceutical composition is to be administered intradermally.

9. The pharmaceutical composition of claim 8, wherein the pharmaceutical composition is to be administered by tattooing, microneedling and/or microneedle patches.

Patentansprüche

1. Oligonukleotid, umfassend einen Sinnstrang und einen Gegensinnstrang, wobei das Oligonukleotid

    einen Sinnstrang umfasst, umfassend eine Nukleotidsequenz
    5’ GACGCUGACCCUGAA$_{m}$GUUCAUCUU 3’; und
    einen Gegensinnstrang umfasst, umfassend eine Nukleotidsequenz
    3’ CUGmCGACUGGGACUUCAAGUAGAA 5’;

    wobei das Nukleotid mit dem tiefgestellten m 2’-O-methyliert ist.

2. Oligonukleotid nach Anspruch 1, wobei der Sinnstrang und/oder der Gegensinnstrang weiterhin eine Phosphothioatbindung umfasst, und wobei das Oligonukleotid

    einen Sinnstrang umfasst, umfassend eine Nukleotidsequenz
    5’ GACGCUGACCCUGAA$_{m}$GUUCAUCUU 3’, und
    einen Gegensinnstrang umfasst, umfassend eine Nukleotidsequenz
    3’ CUG$_{m}$CGACUGGGACUUCAAGUAG$_{PTO}$A$_{PTO}$A 5’; oder
    einen Sinnstrang umfasst, umfassend eine Nukleotidsequenz
    5’ G$_{PTO}$A$_{PTO}$CGCUGACCCUGAA$_{m}$GUUCAUCUU 3’, und
    einen Gegensinnstrang umfasst, umfassend eine Nukleotidsequenz
    3’ CUGmCGACUGGGACUUCAAGUAGAA 5’; oder
    einen Sinnstrang umfasst, umfassend eine Nukleotidsequenz
    5’ G$_{PTO}$A$_{PTO}$CGCUGACCCUGAA$_{m}$GUUCAUCUU 3’, und
    einen Gegensinnstrang umfasst, umfassend eine Nukleotidsequenz
    3’ CUG$_{m}$CGACUGGGACUUCAAGUAG$_{PTO}$A$_{PTO}$A 5’;

    wobei

    wobei das Nukleotid mit dem tiefgestellten m 2’-O-methyliert ist; und
    der Index PTO zwischen zwei Nukleotiden anzeigt, dass die zwei Nukleotide durch eine Phosphothioatbindung verknüpft sind.

3. Oligonukleotid nach Anspruch 1, wobei der Sinnstrang und der Gegensinnstrang weiterhin eine Phosphothioatbindung und 2’-Fluor-Modifikation umfassen und wobei das Oligonukleotid

    einen Sinnstrang umfasst, umfassend eine Nukleotidsequenz
    5’ G$_{PTO}$A$_{PTO}$CGCUG$_{f}$ACCCUGAA$_{m}$GUUCAUCU$_{f}$U 3’, und
    einen Gegensinnstrang umfasst, umfassend eine Nukleotidsequenz
    3’ CUG$_{m}$CGACUGGGAC$_{f}$UUCAAGUAG$_{PTO}$A$_{PTO}$A 5’; oder
    einen Sinnstrang umfasst, umfassend eine Nukleotidsequenz
    5’ G$_{PTO}$A$_{PTO}$CGCUG$_{f}$ACCCUGAA$_{m}$GUUCAUC$_{PTO}$U$_{fPTO}$U 3’, und
    einen Gegensinnstrang umfasst, umfassend eine Nukleotidsequenz
    3’ CUG$_{m}$CGACUGGGAC$_{f}$UUCAAGUAG$_{PTO}$A$_{PTO}$A 5’; oder
    einen Sinnstrang umfasst, umfassend eine Nukleotidsequenz
    5’ GACGCUG$_{f}$ACCCUGAA$_{m}$GUUCAUC$_{PTO}$U$_{fPTO}$U 3’, und
    einen Gegensinnstrang umfasst, umfassend eine Nukleotidsequenz
    3’ CUG$_{m}$CGACUGGGAC$_{f}$UUCAAGUAG$_{PTO}$A$_{PTO}$A 5’;

    wobei

    das Nukleotid mit dem tiefgestellten m 2’-O-methyliert ist;
    das Nukleotid mit dem tiefgestellten f 2’-Fluor ist; und
    der Index PTO zwischen zwei Nukleotiden anzeigt, dass die zwei Nukleotide durch eine Phosphothioatbindung verknüpft sind.

4. Oligonukleotid nach einem beliebigen der Ansprüche 1 bis 3, wobei das Oligonukleotid ein Ligand für RIG-I ist.

5. Pharmazeutische Zusammensetzung, umfassend ein Oligonukleotid gemäß einem beliebigen der Ansprüche 1 bis 3.

6. Pharmazeutische Zusammensetzung nach Anspruch 5, wobei die pharmazeutische Zusammensetzung systemisch oder lokal zu verabreichen ist.

7. Pharmazeutische Zusammensetzung nach Anspruch 6, wobei die pharmazeutische Zusammensetzung intraperitoneal, intramuskulär, intravenös, intranasal, subkutan, intradermal, oder intrathekal zu verabreichen ist.

8. Pharmazeutische Zusammensetzung nach Anspruch 7, wobei die pharmazeutische Zusammensetzung intradermal zu verabreichen ist.

9. Pharmazeutische Zusammensetzung nach Anspruch 8, wobei die pharmazeutische Zusammensetzung intradermal durch Tätowieren, Mikronadeln und/oder Mikronadelpflaster zu verabreichen ist.

Revendications

1. Oligonucléotide comprenant un brin sens et un brin antisens, dans lequel l'oligonucléotide comprend

un brin sens comprenant la séquence nucléotidique
5' $GACGCUGACCCUGAA_{m}GUUCAUCUU$ 3' ; et
un brin antisens comprenant la séquence nucléotidique
3' CUGmCGACUGGGACUUCAAGUAGAA 5' ;

dans lequel le nucléotide à index m est 2'-O-méthylé.

2. Oligonucléotide selon la revendication 1, dans lequel le brin sens et/ou le brin antisens comprend en outre une liaison phosphothioate, et dans lequel l'oligonucléotide comprend

un brin sens comprenant la séquence nucléotidique
5' $GACGCUGACCCUGAA_{m}GUUCAUCUU$ 3' ; et
un brin antisens comprenant la séquence nucléotidique
3' $CUG_{m}CGACUGGGACUUCAAGUAG_{PTO}A_{PTO}A$ 5' ; ou
un brin sens comprenant la séquence nucléotidique
5' $G_{PTO}A_{PTO}CGCUGACCCUGAA_{m}GUUCAUCUU$ 3' ; et
un brin antisens comprenant la séquence nucléotidique
3' CUGmCGACUGGGACUUCAAGUAGAA 5' ; ou
un brin sens comprenant la séquence nucléotidique
5' $G_{PTO}A_{PTO}CGCUGACCCUGAA_{m}GUUCAUCUU$ 3' ; et
un brin antisens comprenant la séquence nucléotidique
3' $CUG_{m}CGACUGGGACUUCAAGUAG_{PTO}A_{PTO}A$ 5' ;

dans lequel

le nucléotide à index m est 2'-O-méthylé ; et
l'indice PTO entre deux nucléotides indique que lesdits deux nucléotides sont liés par une liaison phosphothioate.

3. Oligonucléotide selon la revendication 1, dans lequel le brin sens et le brin antisens comprennent en outre une liaison phosphothioate et une modification 2'-fluoro, et dans lequel l'oligonucléotide comprend

un brin sens comprenant la séquence nucléotidique
5' $G_{PTO}A_{PTO}CGCUG_{f}ACCCUGAA_{m}GUUCAUCU_{f}U$ 3' ; et
un brin antisens comprenant la séquence nucléotidique
3' $CUG_{m}CGACUGGGAC_{f}UUCAAGUAG_{PTO}A_{PTO}A$ 5' ; ou
un brin sens comprenant la séquence nucléotidique
5' $G_{PTO}A_{PTO}CGCUG_{f}ACCCUGAA_{m}GUUCAUC_{PTO}U_{fPTO}U$ 3' ; et
un brin antisens comprenant la séquence nucléotidique
3' $CUG_{m}CGACUGGGAC_{f}UUCAAGUAG_{PTO}A_{PTO}A$ 5' ; ou

un brin sens comprenant la séquence nucléotidique
5’ GACGCUG$_{f}$ACCCUGAA$_{m}$GUUCAUC$_{PTO}$U$_{fPTO}$U 3’ ; et
un brin antisens comprenant la séquence nucléotidique
3’ CUG$_{m}$CGACUGGGAC$_{f}$UUCAAGUAG$_{PTO}$A$_{PTO}$A 5’ ;

dans lequel

le nucléotide à index m est 2’-O-méthylé ;
le nucléotide à index f est un 2’-fluoro ; et
l’indice PTO entre deux nucléotides indique que lesdits deux nucléotides sont liés par une liaison phosphothioate.

4. Oligonucléotide selon l’une quelconque des revendications 1 à 3, dans lequel l’oligonucléotide est un ligand de RIG-I.

5. Composition pharmaceutique comprenant un oligonucléotide selon l’une quelconque des revendications 1 à 3.

6. Composition pharmaceutique selon la revendication 5, dans laquelle la composition pharmaceutique doit être administrée par voie systémique ou locale.

7. Composition pharmaceutique selon la revendication 6, dans laquelle la composition pharmaceutique doit être administrée par voie intrapéritonéale, intramusculaire, intraveineuse, intranasale, sous-cutanée, intradermique ou intrathécale.

8. Composition pharmaceutique selon la revendication 7, dans laquelle la composition pharmaceutique doit être administrée par voie intradermique.

9. Composition pharmaceutique selon la revendication 8, dans laquelle la composition pharmaceutique doit être administrée par tatouage, micropuncture et/ou des patchs de micro-aiguilles.

Figure 1

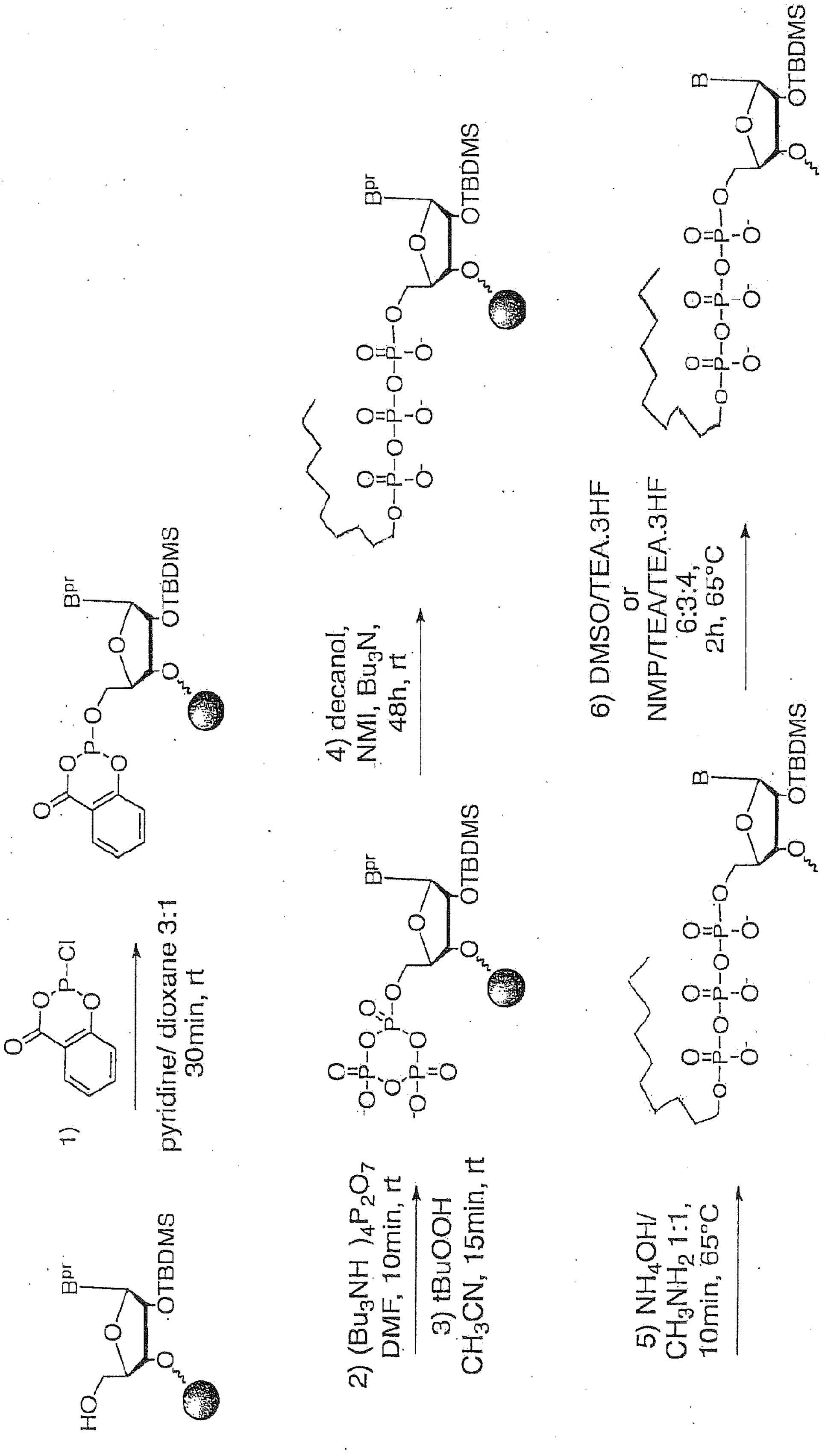

HO
B^pr
OTBDMS
1)
P-Cl
pyridine/ dioxane 3:1
30min, rt
2) (Bu3NH)4P2O7
DMF, 10min, rt
3) tBuOOH
CH3CN, 15min, rt
4) decanol,
NMI, Bu3N,
48h, rt
5) NH4OH/
CH3NH2 1:1,
10min, 65°C
6) DMSO/TEA.3HF
or
NMP/TEA/TEA.3HF
6:3:4,
2h, 65°C
B

Figure 2

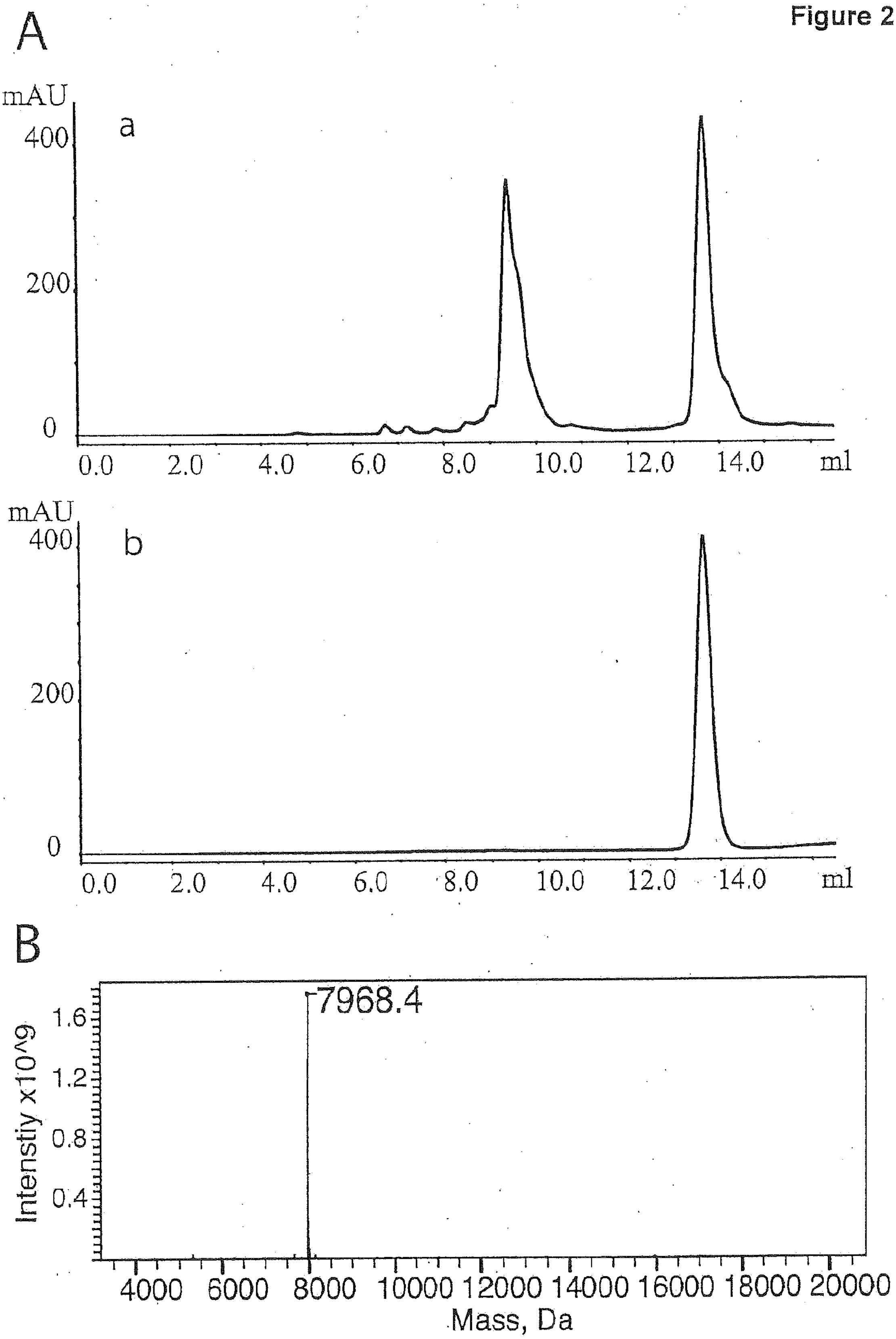

A
mAU
400
a
200
0
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
ml
mAU
400
b
200
0
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
ml
B
7968.4
Intenstiy x10^9
1.6
1.2
0.8
0.4
4000
6000
8000
10000
12000
14000
16000
18000
20000
Mass, Da

Figure 3

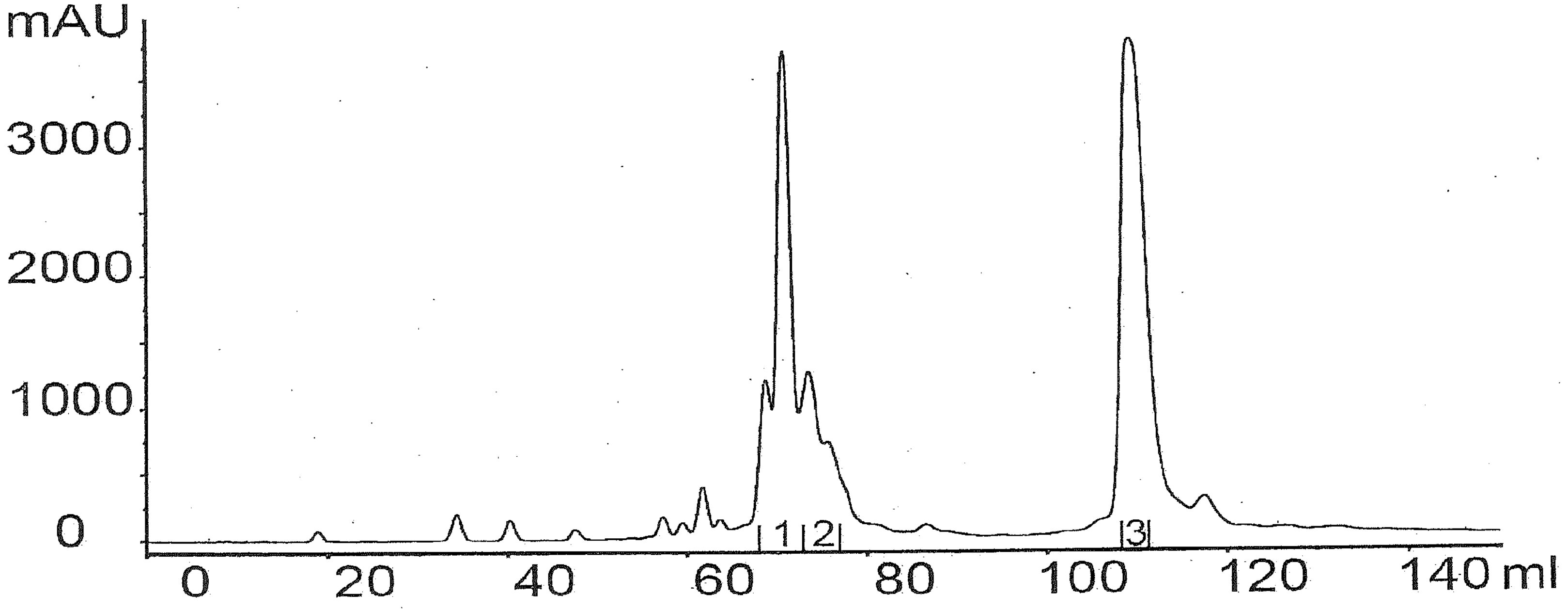


Figure 4

$$-O-\left(CH_2-CH_2-O\right)_n-CH_2-CH_2-$$

$$-O-\left(\left(CH_2\right)_m-CH_2-O\right)_n-CH_2-CH_2-$$

$$-O-\left(\left(CHR\right)_m-CH_2-O\right)_n-CH_2-CH_2-$$

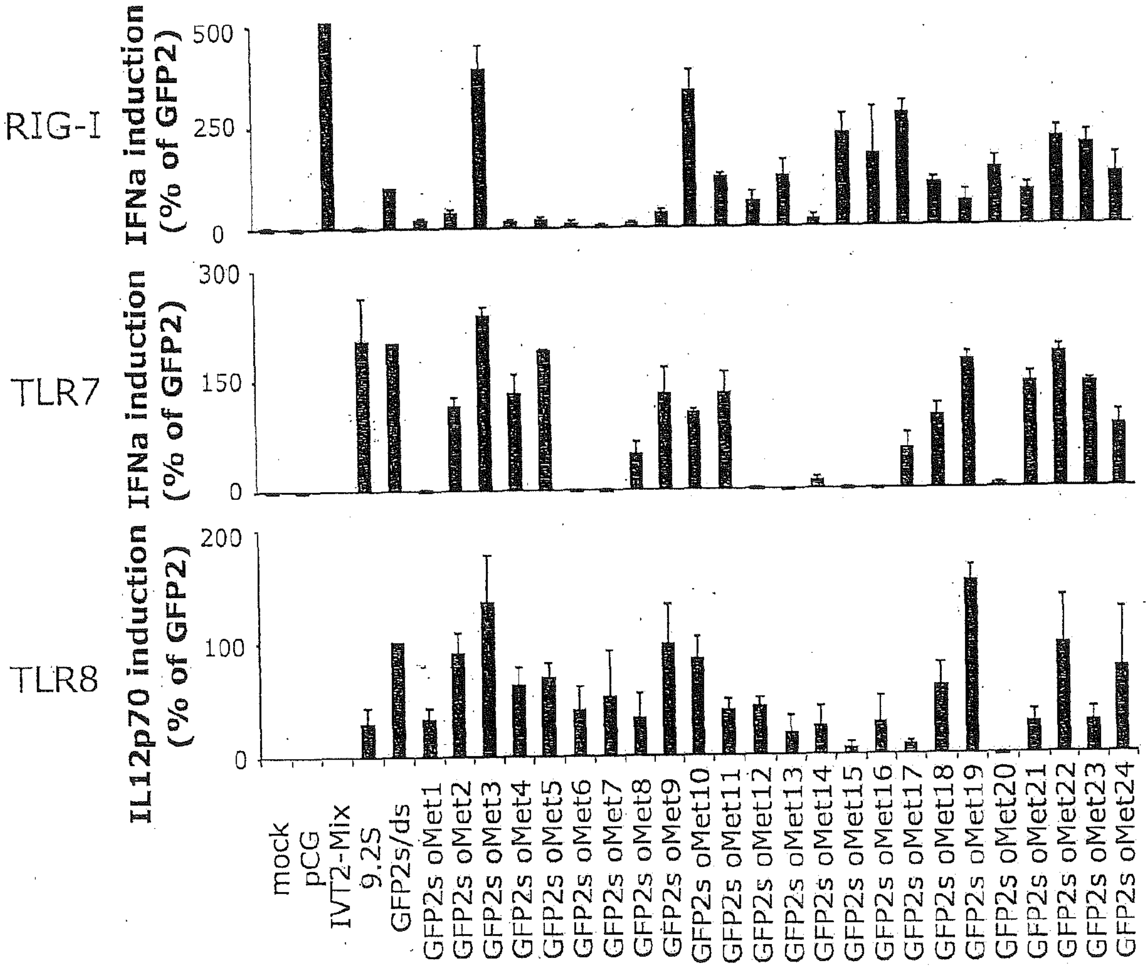
$$-O-\left(CH_2-CH_2-CH_2-O\right)_n-CH_2-CH_2-$$

$$-O-\left(\left(CH_2\right)_m-CH_2-CH_2-O\right)_n-CH_2-CH_2-$$

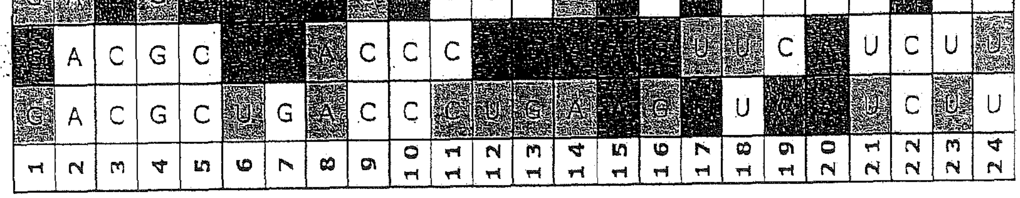
$$-O-\left(\left(CHR\right)_m-CH_2-CH_2-O\right)_n-CH_2-CH_2-$$

Figure 5

A GFP2 sense strand (5'-3')



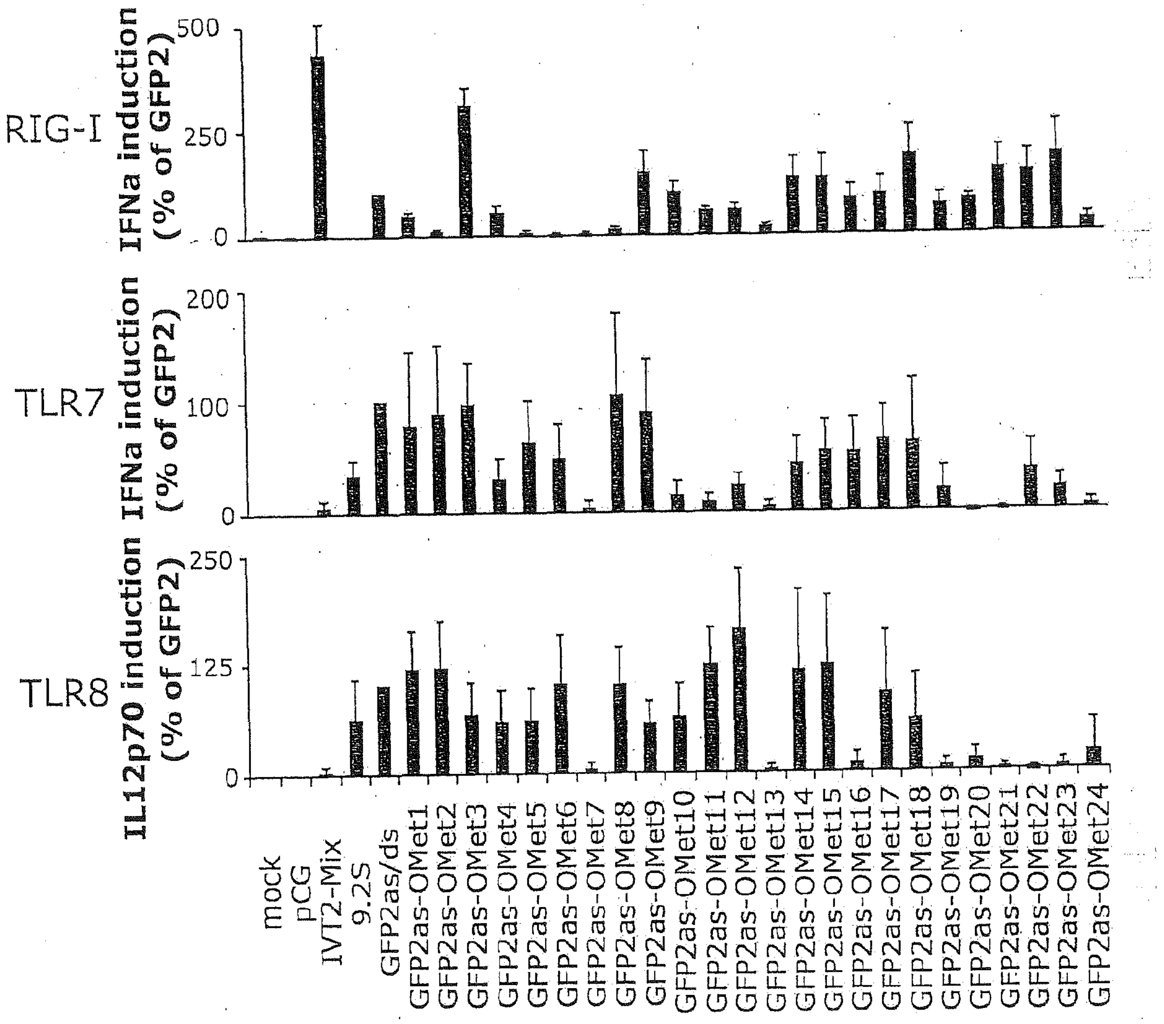
GFP2 sense strand (5'-3')



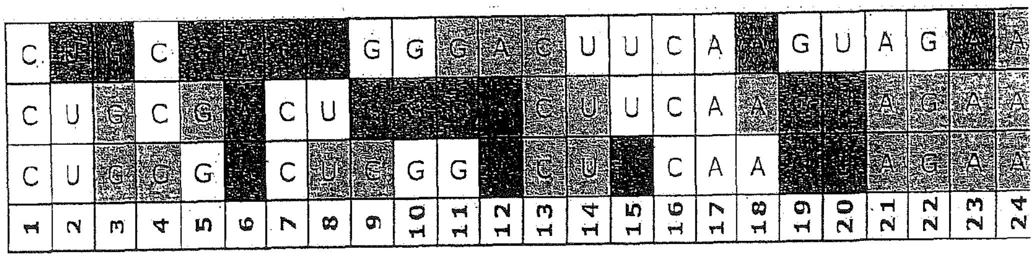
G	A	[illegible]	G	[illegible]	[illegible]	[illegible]	[illegible]	G	[illegible]	C	U	G	A	[illegible]	G	[illegible]	U	C	A	U	[illegible]	U	U
[illegible]	A	C	G	C	[illegible]	[illegible]	A	C	C	C	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	U	U	C	[illegible]	U	C	U	U
G	A	C	G	C	U	G	A	C	C	C	U	G	A	[illegible]	G	[illegible]	U	[illegible]	[illegible]	U	C	U	U
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24

Figure 5 continued

B GFP2 antisense strand (3'-5')



GFP2 antisense strand (3'-5')



C	[illegible]	[illegible]	C	[illegible]	[illegible]	[illegible]	[illegible]	G	G	G	A	C	U	U	C	A	A	G	U	A	G	A	A
C	U	G	C	G	[illegible]	C	U	[illegible]	[illegible]	[illegible]	[illegible]	C	U	U	C	A	A	[illegible]	[illegible]	A	G	A	A
C	U	G	G	G	[illegible]	C	U	C	G	G	[illegible]	C	U	[illegible]	C	A	A	[illegible]	[illegible]	A	G	A	A
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24

Figure 6

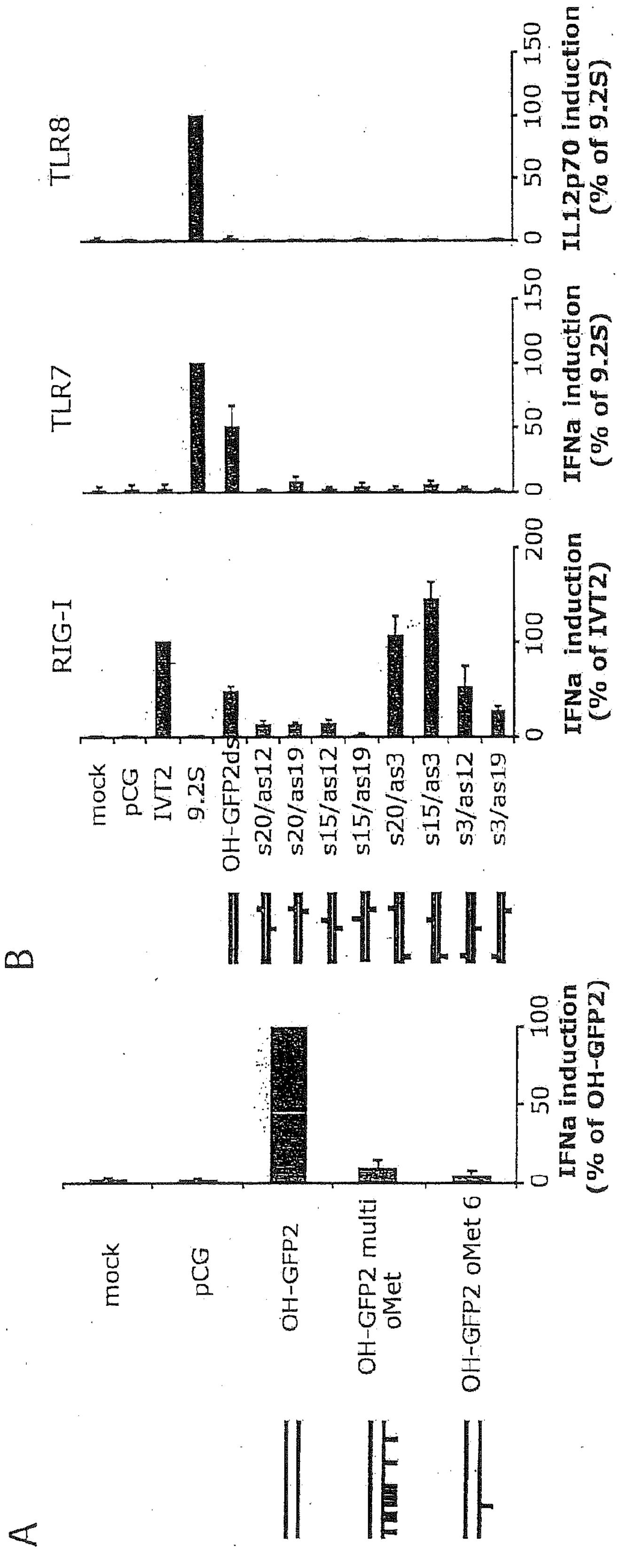

A
mock
pCG
OH-GFP2
OH-GFP2 multi oMet
OH-GFP2 oMet 6
0
50
100
IFNa induction (% of OH-GFP2)
B
RIG-I
TLR7
TLR8
mock
pCG
IVT2
9.2S
OH-GFP2ds
s20/as12
s20/as19
s15/as12
s15/as19
s20/as3
s15/as3
s3/as12
s3/as19
0
100
200
IFNa induction (% of IVT2)
0
50
100
150
IFNa induction (% of 9.2S)
0
50
100
150
IL12p70 induction (% of 9.2S)

Figure 7

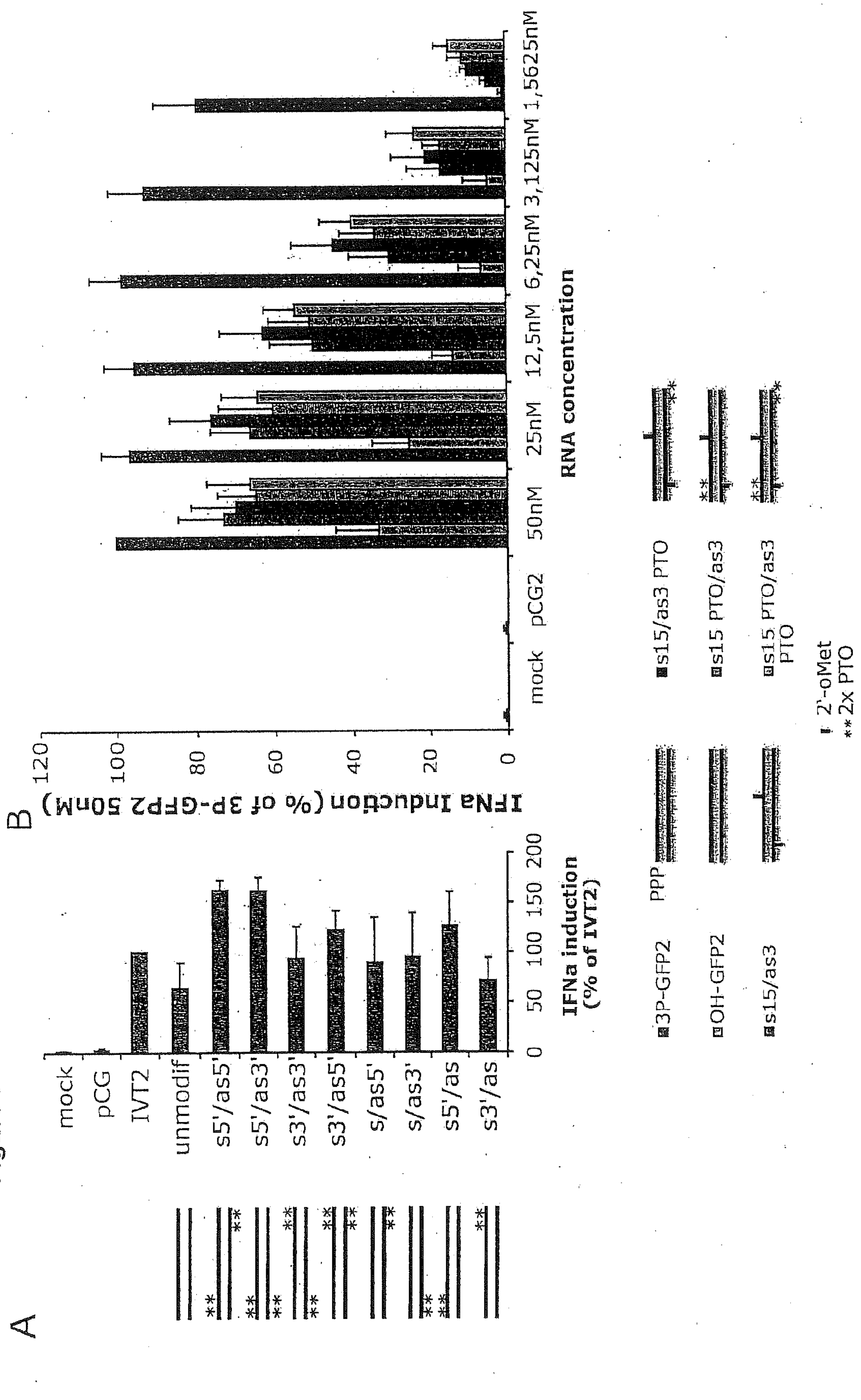

A
mock
pCG
IVT2
unmodif
s5'/as5'
s5'/as3'
s3'/as3'
s3'/as5'
s/as5'
s/as3'
s5'/as
s3'/as
0
50
100
150
200
IFNa induction
(% of IVT2)
B
IFNa Induction (% of 3P-GFP2 50nM)
0
20
40
60
80
100
120
mock
pCG2
50nM
25nM
12,5nM
6,25nM
3,125nM
1,5625nM
RNA concentration
3P-GFP2
OH-GFP2
s15/as3
PPP
s15/as3 PTO
s15 PTO/as3
s15 PTO/as3 PTO
2'-oMet
** 2x PTO

Figure 8

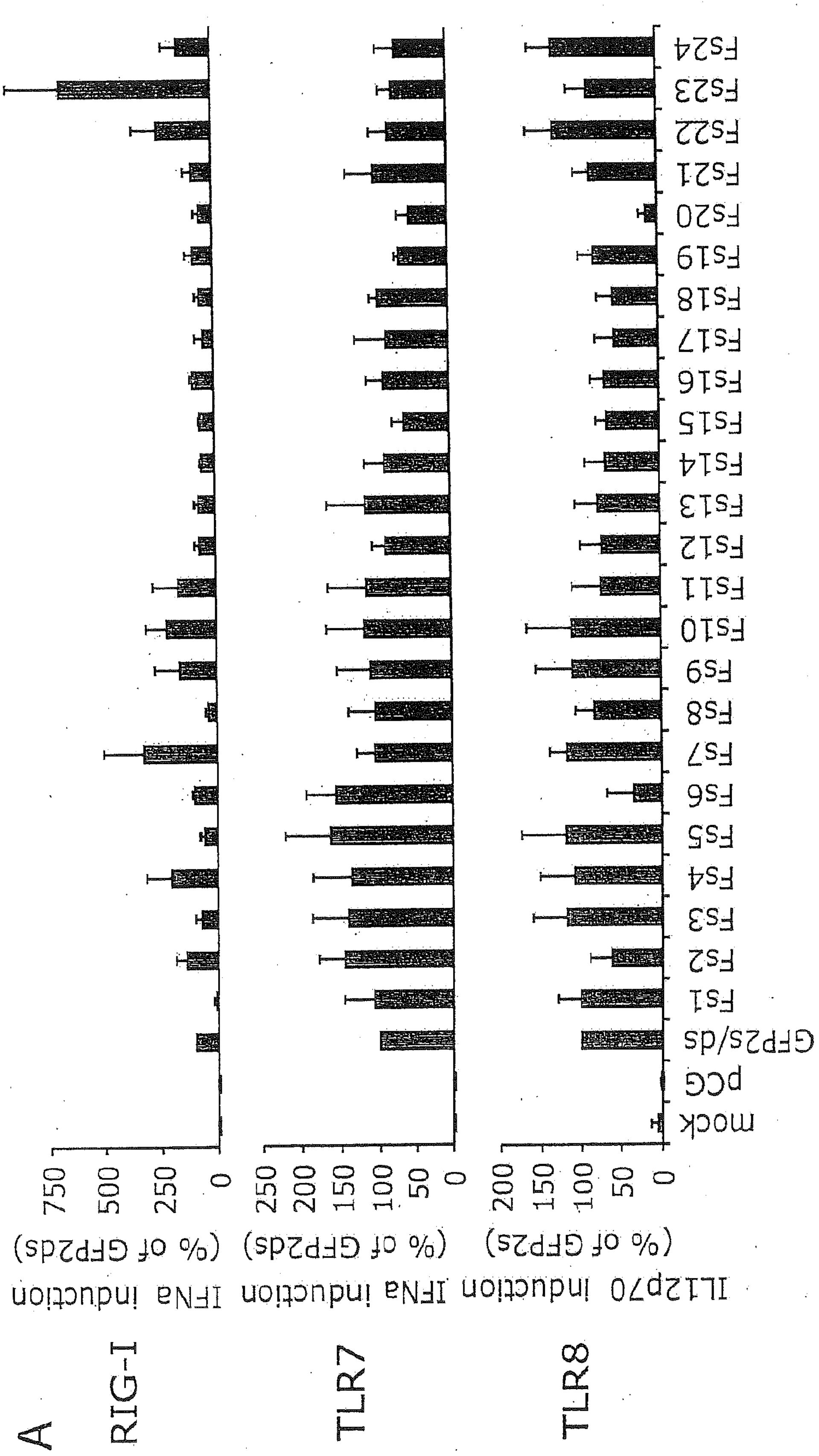

A
RIG-I
TLR7
TLR8
IFNa induction (% of GFP2ds)
750
500
250
0
IFNa induction (% of GFP2ds)
250
200
150
100
50
0
IL12p70 induction (% of GFP2s)
200
150
100
50
0
mock
pCG
GFP2s/ds
Fs1
Fs2
Fs3
Fs4
Fs5
Fs6
Fs7
Fs8
Fs9
Fs10
Fs11
Fs12
Fs13
Fs14
Fs15
Fs16
Fs17
Fs18
Fs19
Fs20
Fs21
Fs22
Fs23
Fs24

Figure 8 continued

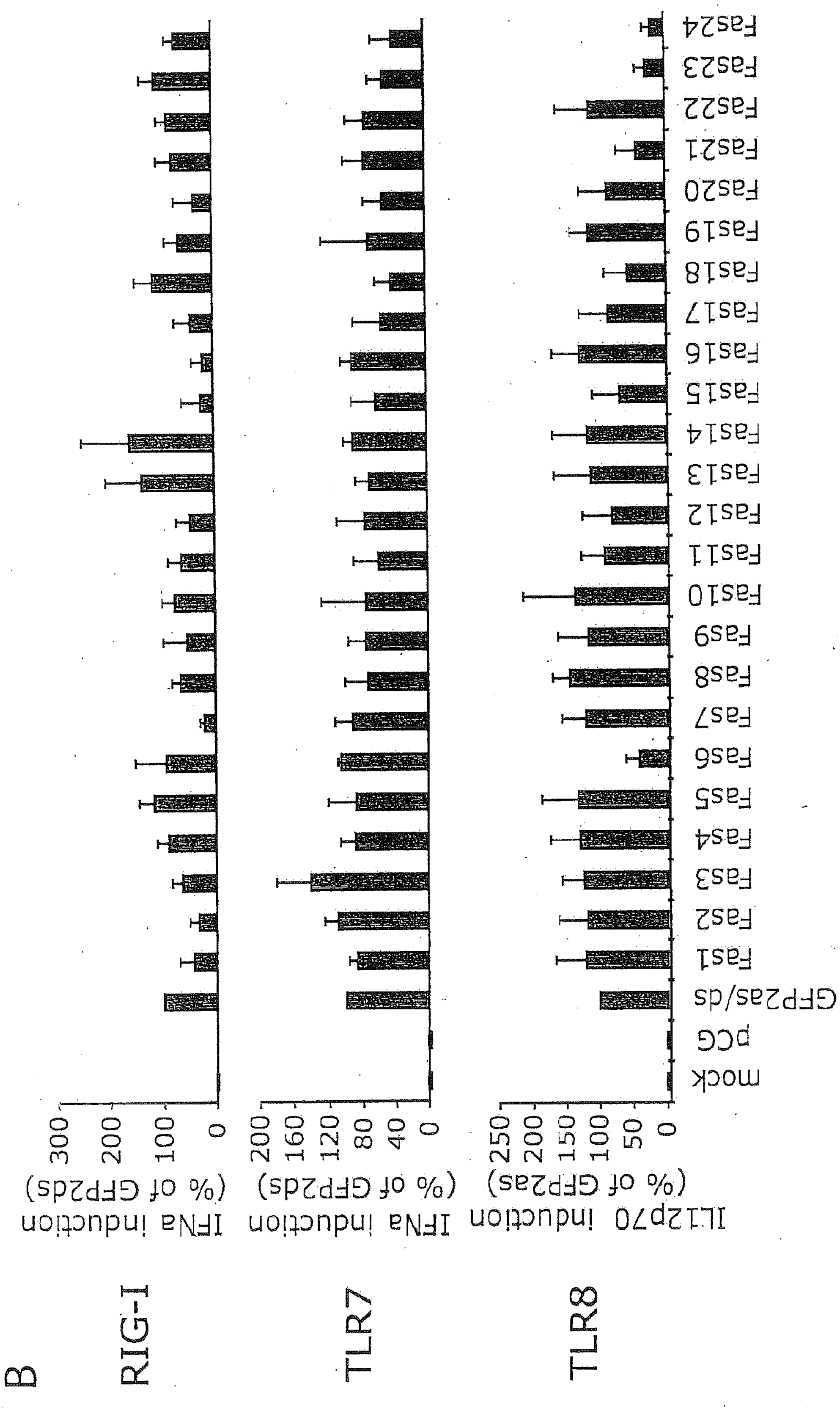


Figure 9

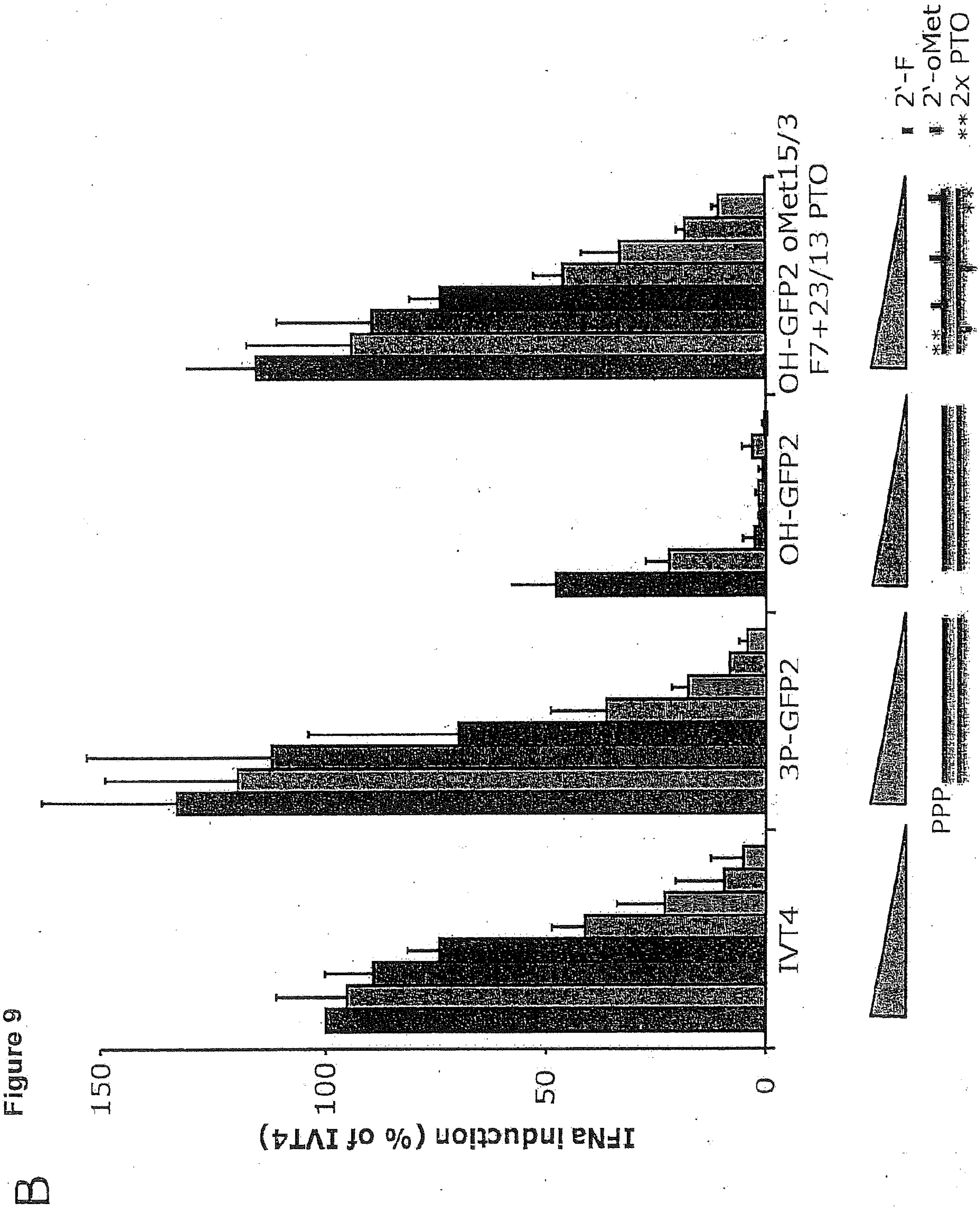

B
IFNa induction (% of IVT4)
150
100
50
0
IVT4
3P-GFP2
OH-GFP2
OH-GFP2 oMet15/3
F7+23/13 PTO
PPP
**
2`-F
2`-oMet
** 2x PTO

Figure 9 continued

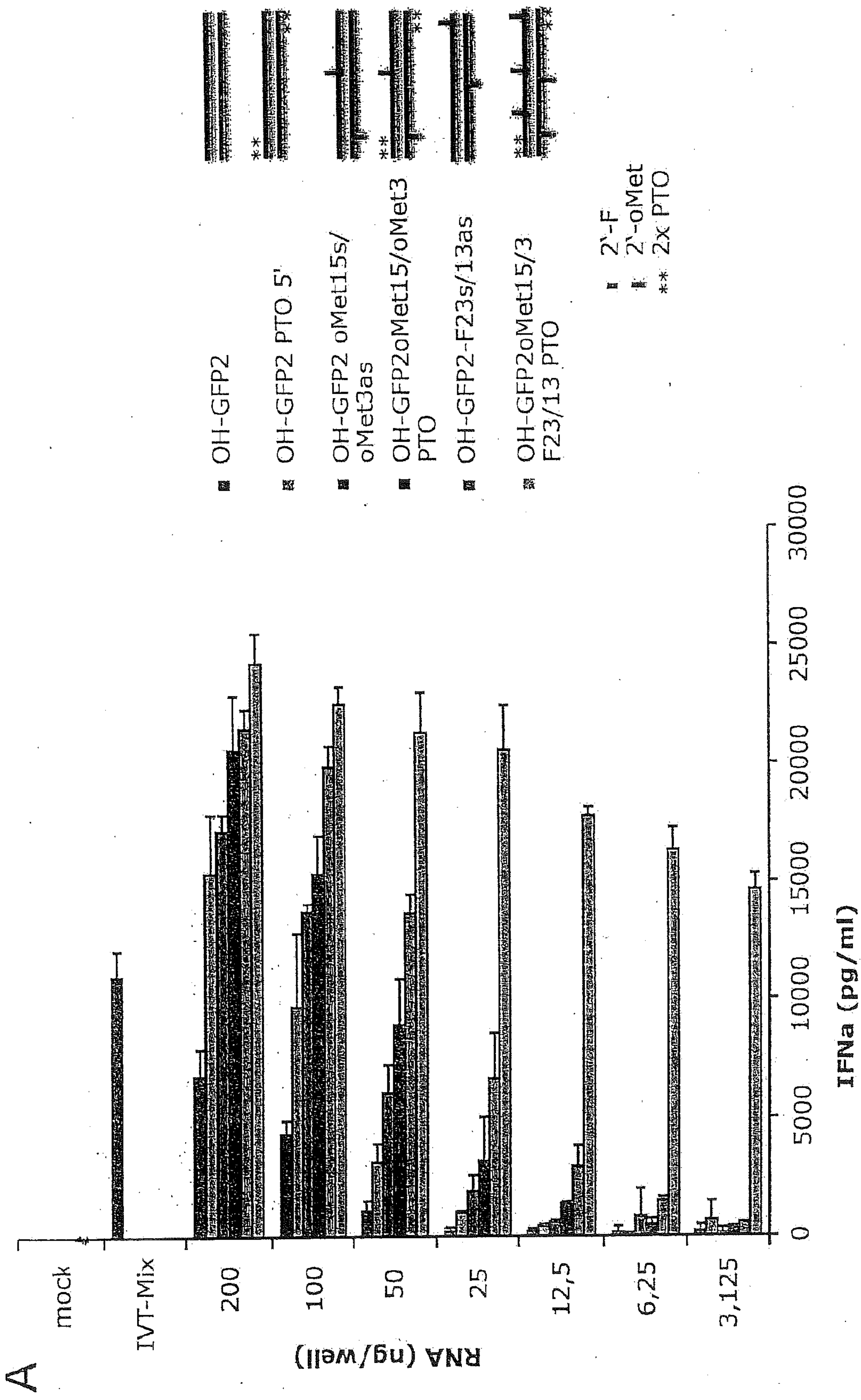


Figure 10

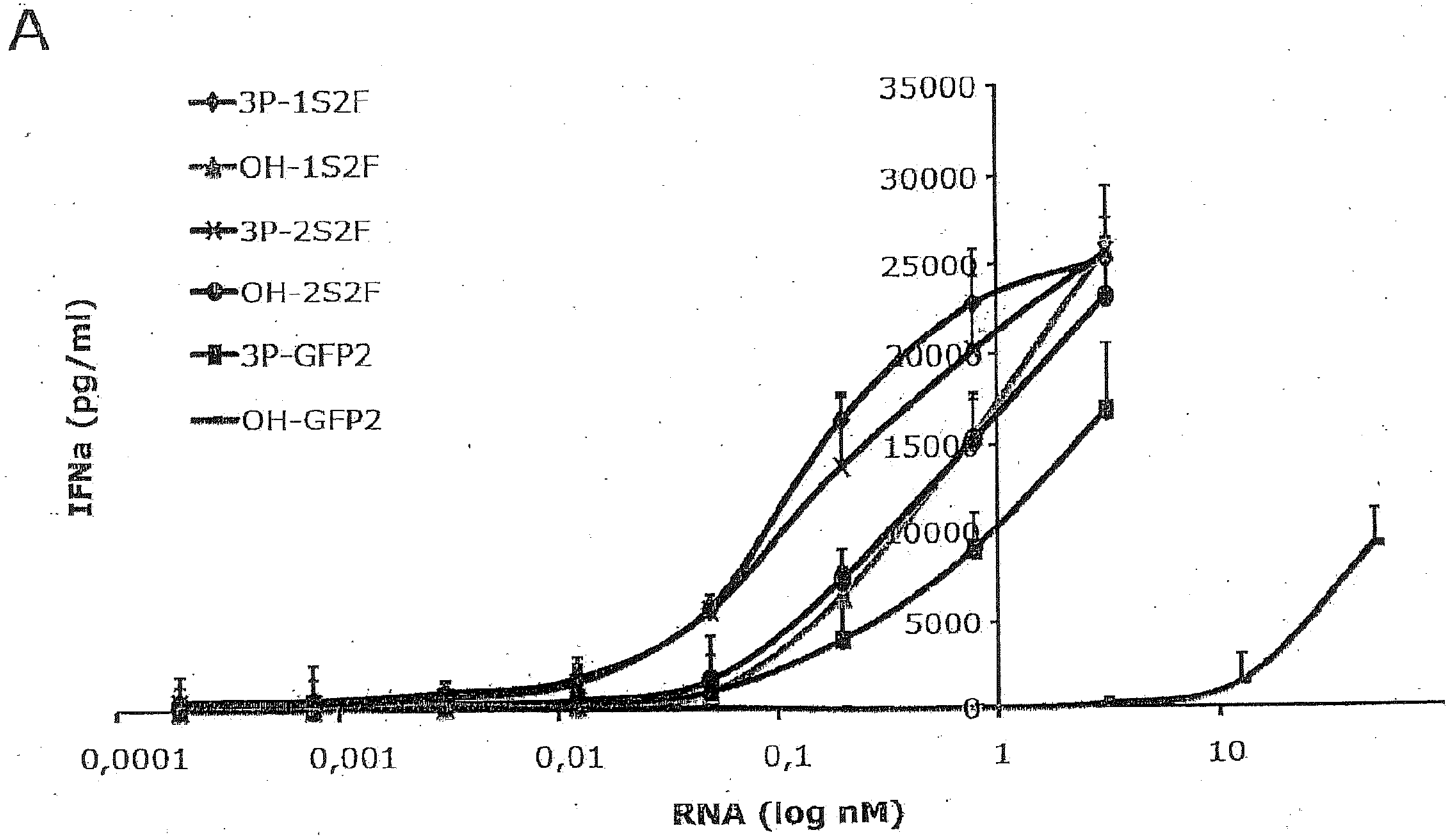

A
3P-1S2F
OH-1S2F
3P-2S2F
OH-2S2F
3P-GFP2
OH-GFP2
IFNa (pg/ml)
35000
30000
25000
20000
15000
10000
5000
0
0,0001
0,001
0,01
0,1
1
10
RNA (log nM)

Figure 10 continued

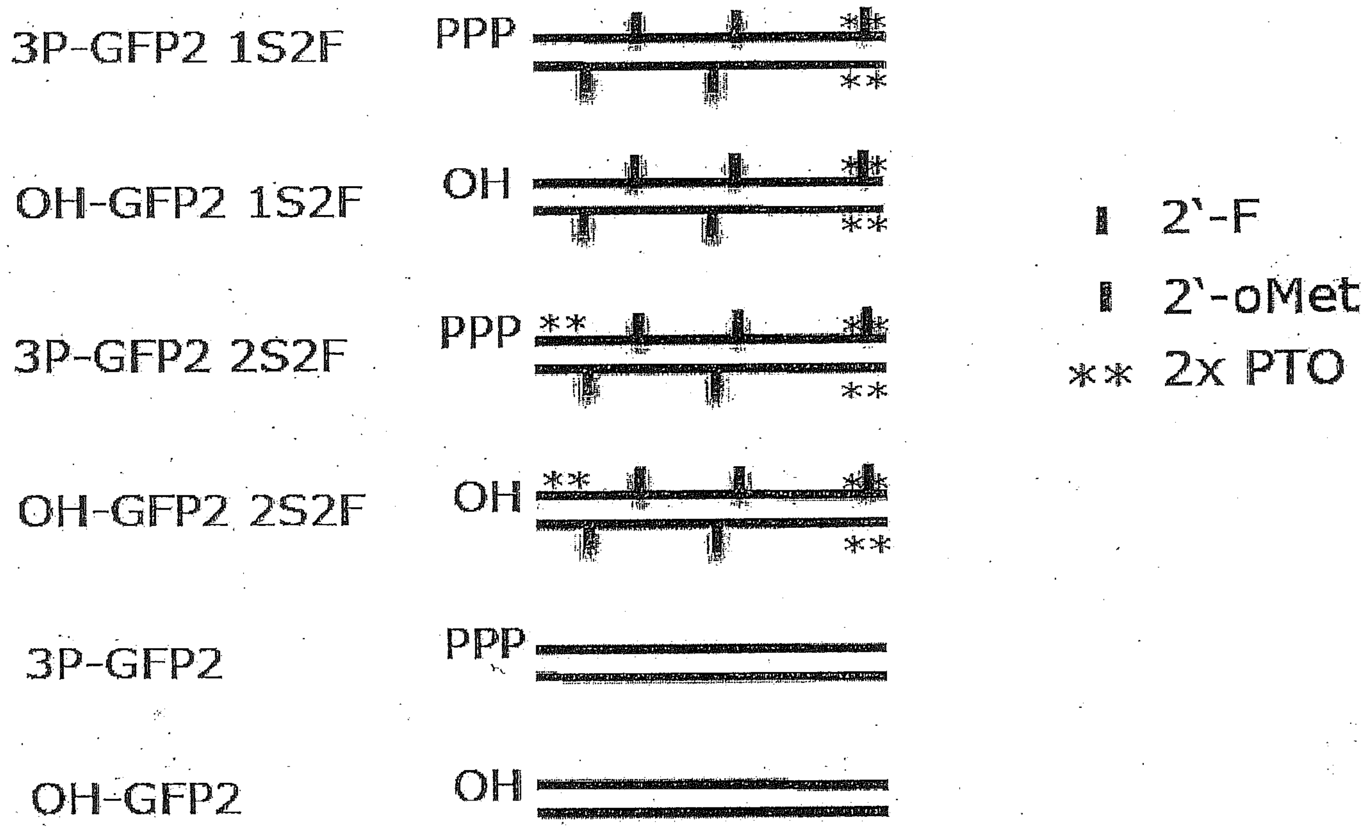


Figure 10 continued

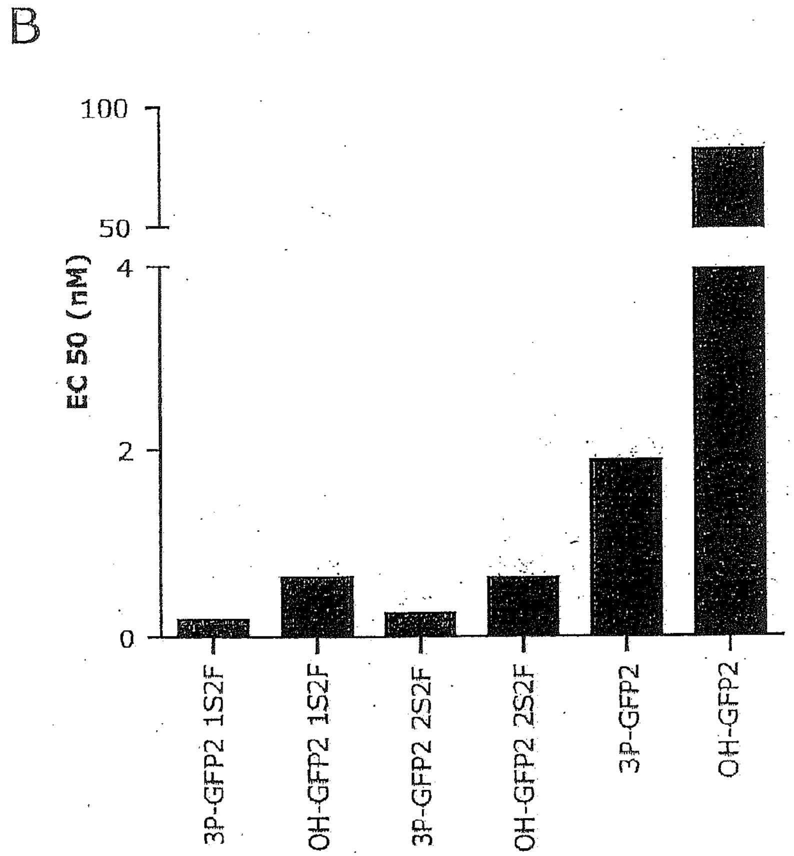
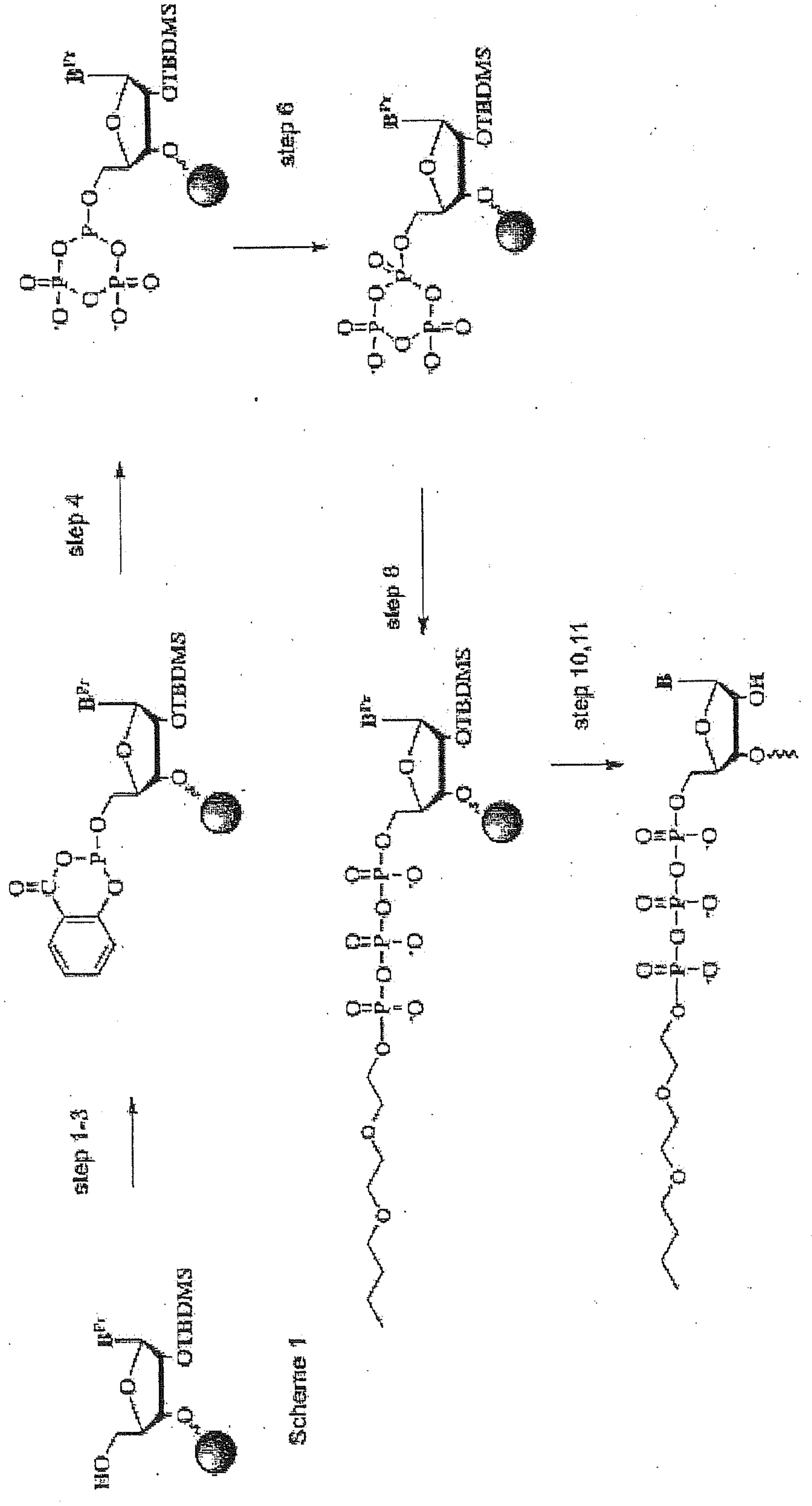


Figure 11



Scheme 1

Figure 12

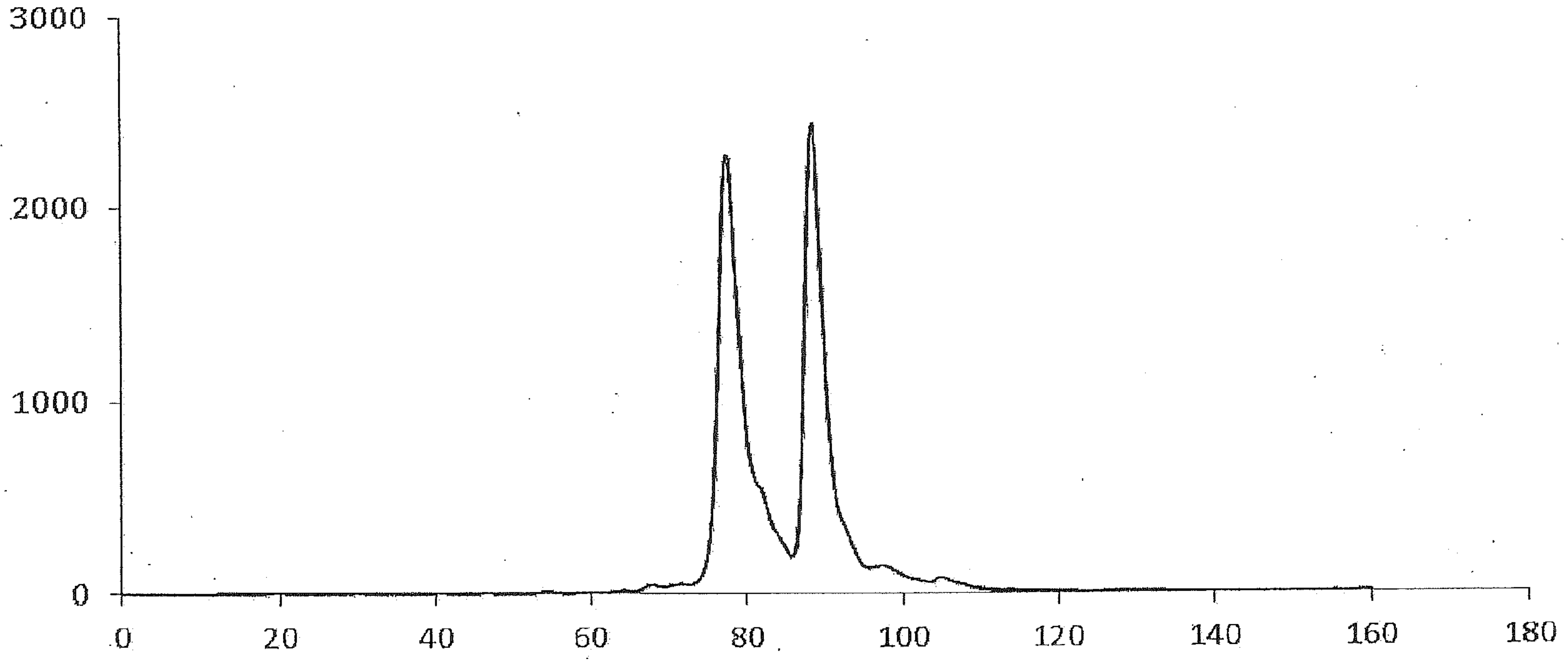


Figure 13

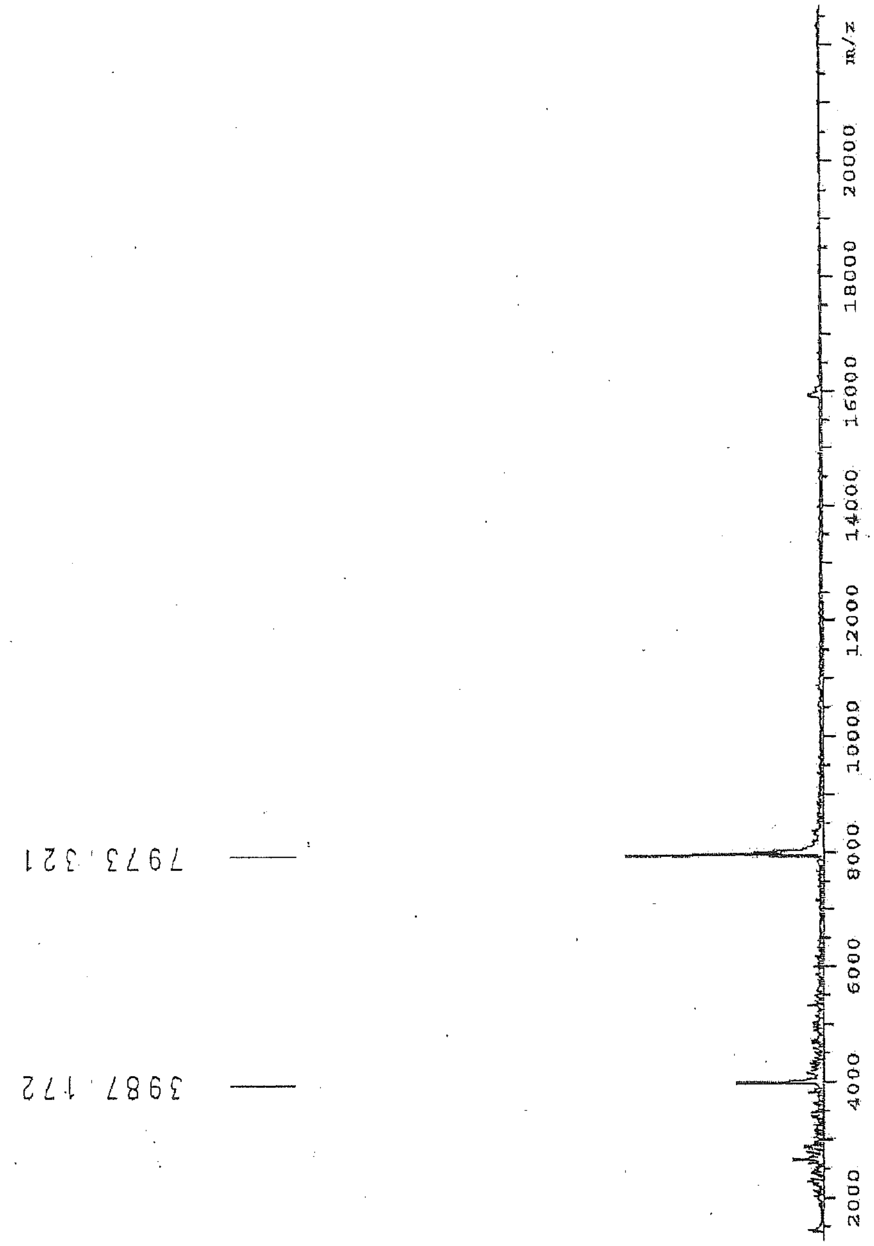

7973.321
3987.172
2000
4000
6000
8000
10000
12000
14000
16000
18000
20000
m/z

Figure 14

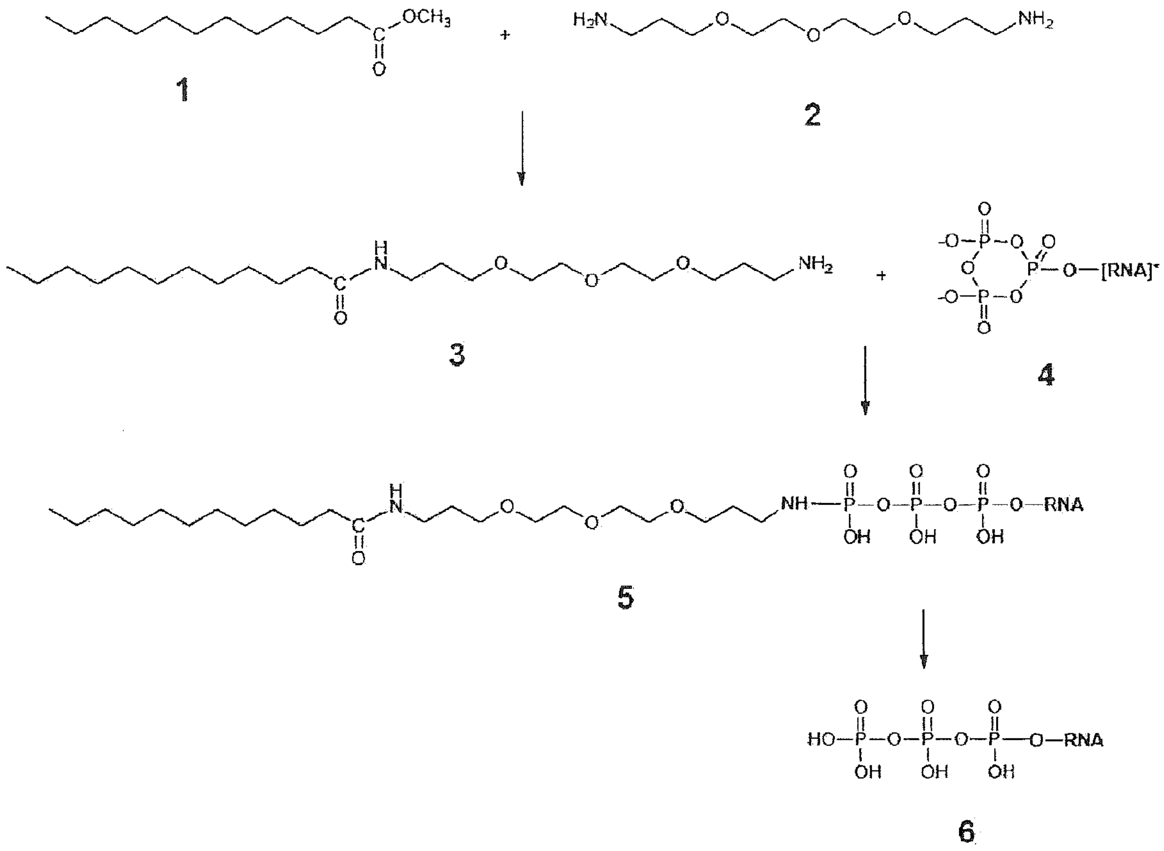


Figure 15

A

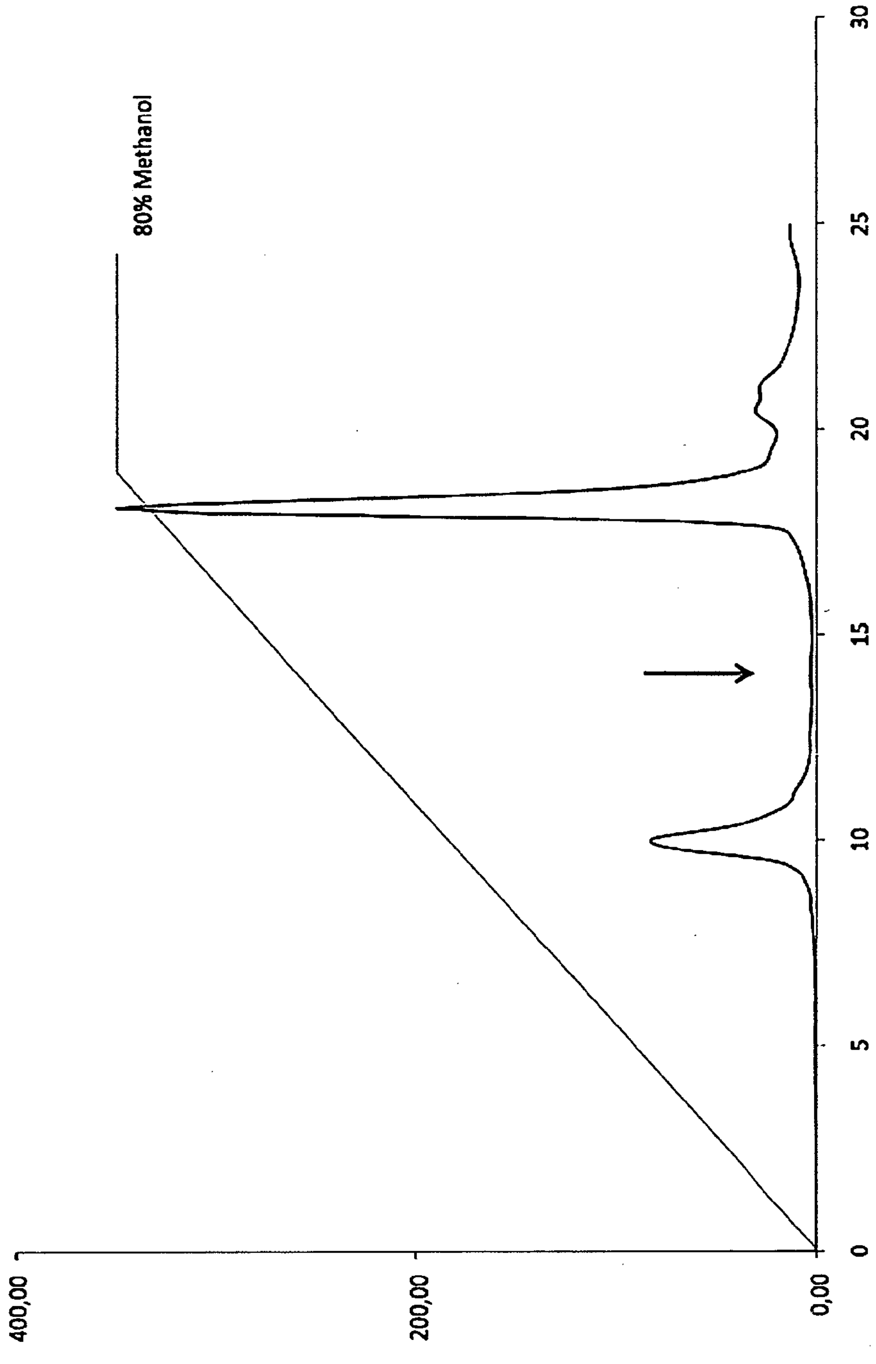


Figure 15 continued

B

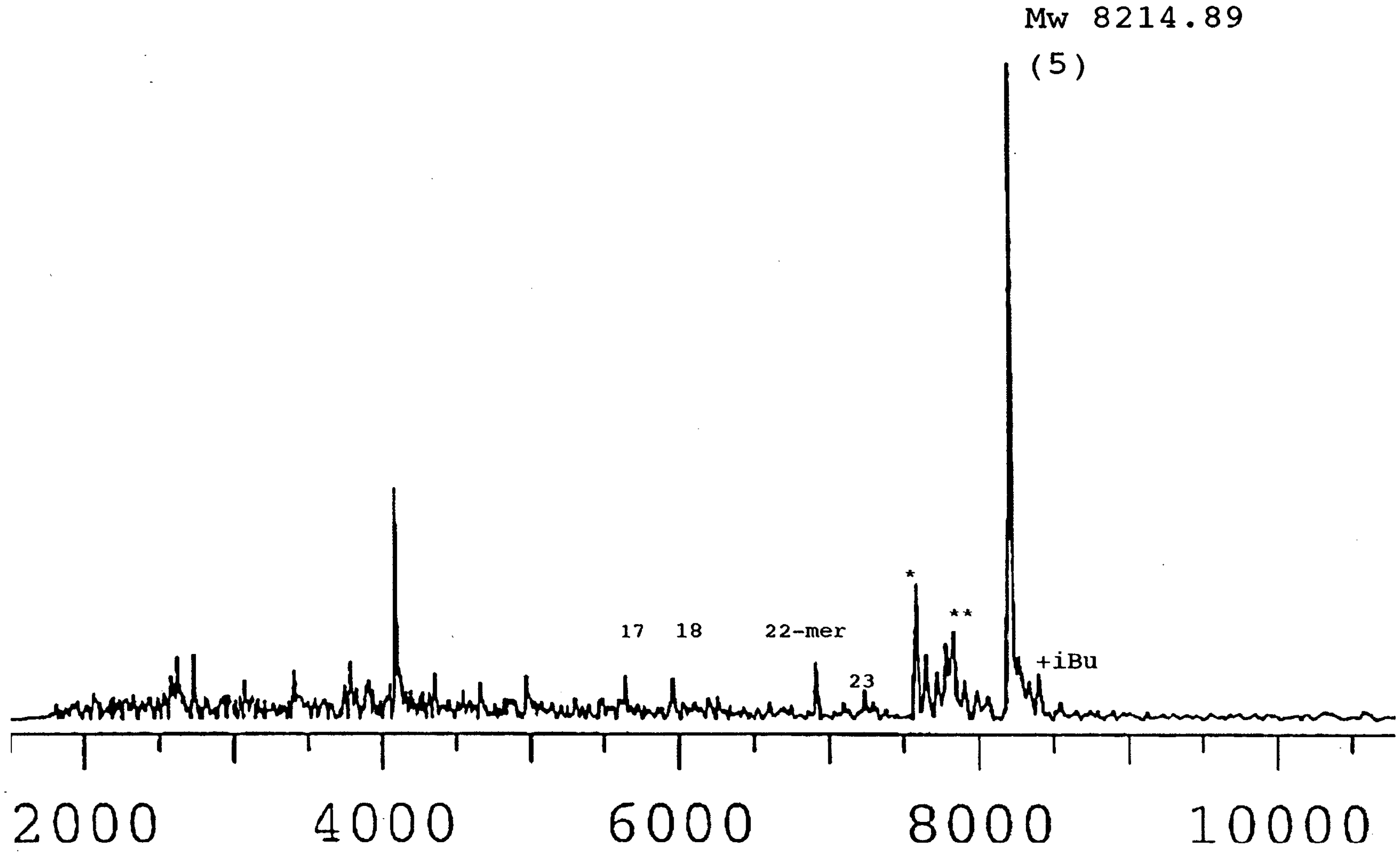
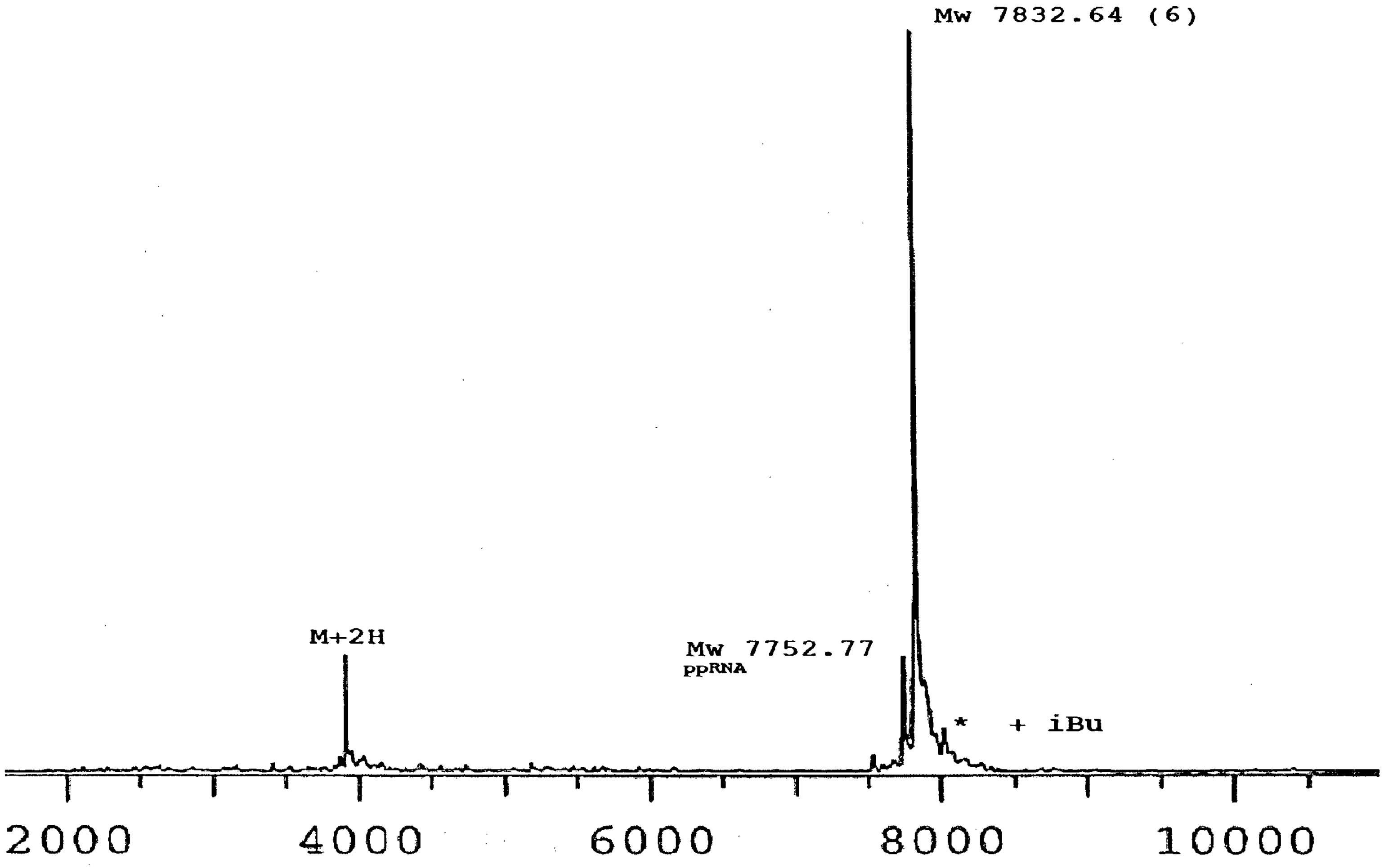


Figure 15 continued

C



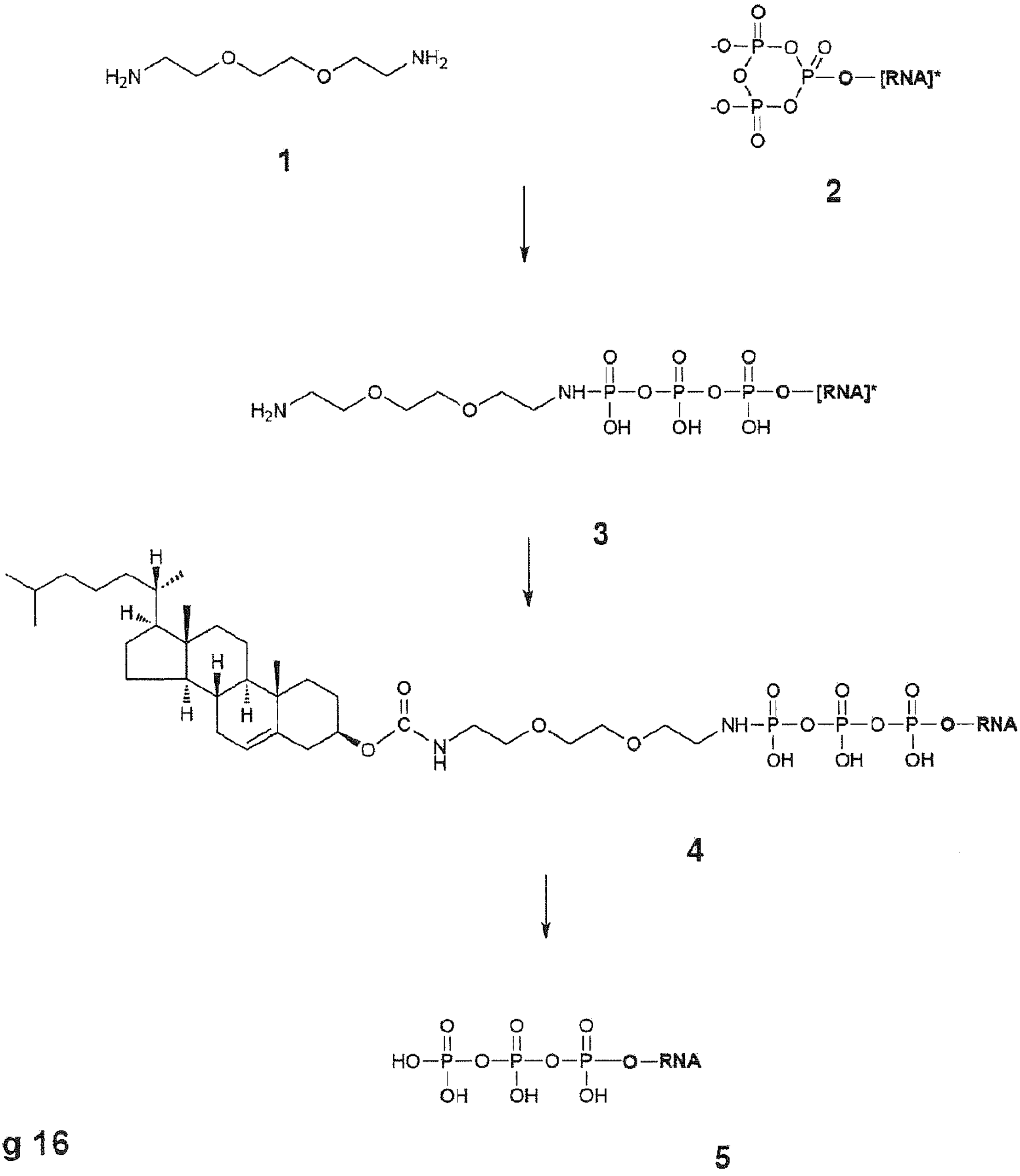
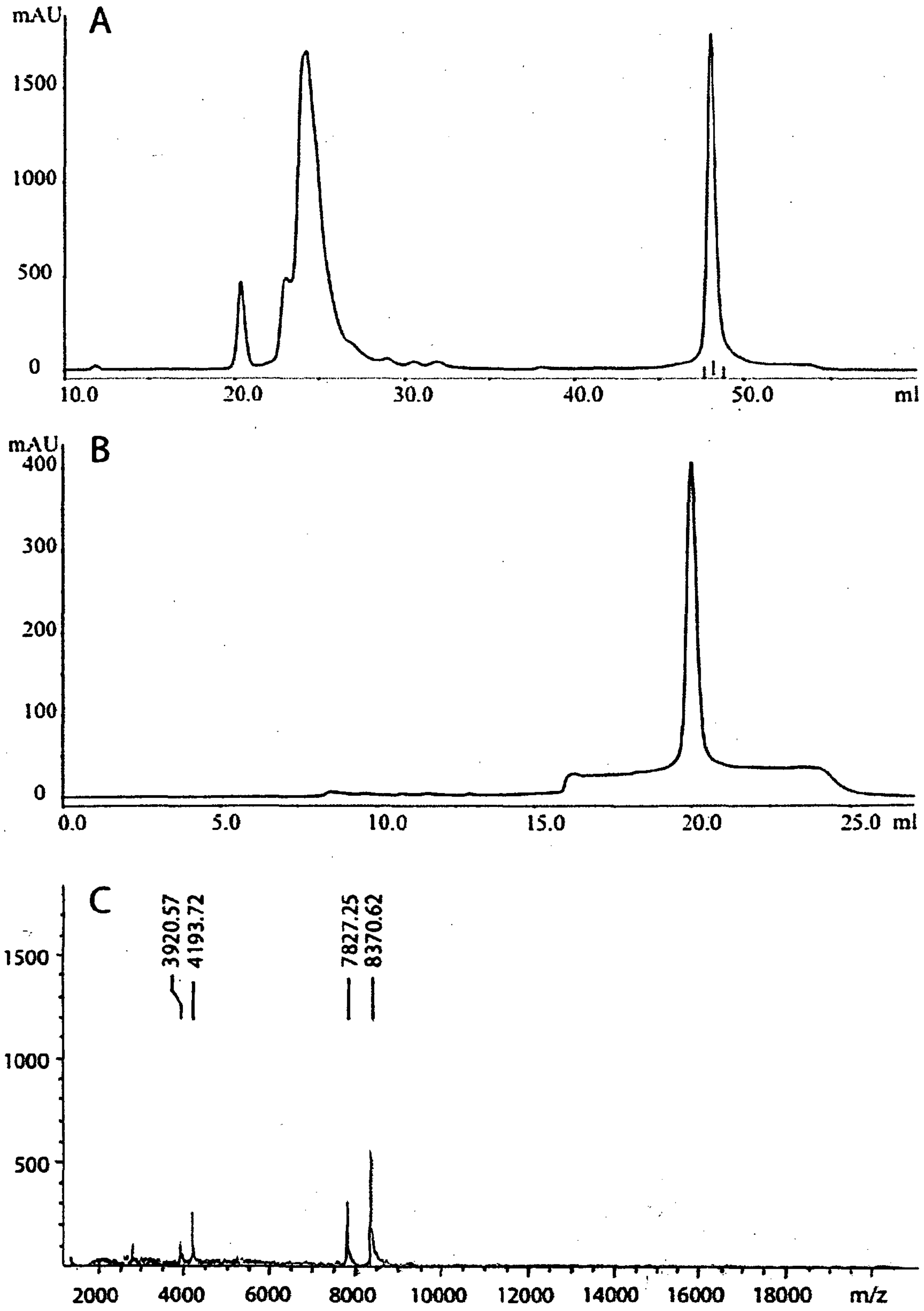


Fig 16

Figure 17


A
mAU
1500
1000
500
0
10.0
20.0
30.0
40.0
50.0
ml
B
mAU
400
300
200
100
0
0.0
5.0
10.0
15.0
20.0
25.0
ml
C
3920.57
4193.72
7827.25
8370.62
1500
1000
500
2000
4000
6000
8000
10000
12000
14000
16000
18000
m/z

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- US 6900308 B2 **[0003]**
- US 7285658 B **[0003]**
- US 7598230 B **[0003]**
- US 7807653 B **[0003]**
- WO 9640159 A **[0004] [0006] [0068]**
- WO 2009060281 A **[0005] [0006] [0068]**
- EP 12186444 A **[0140]**

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- **SCHLEE et al.** *Immunity,* 2009, vol. 31, 25-34 **[0002]**
- **LUDWIG J et al.** *J. Org. Chem.,* 1989, vol. 54, 631-635 **[0003]**
- **GAUR R.K. et al.** *Tetrahedron Letters,* 1992, vol. 33, 3301-3304 **[0003]**
- **WANG Y et al.** *Nature Structural & Molecular Biology,* 2010, vol. 17 (7), 781-787 **[0006]**